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Some aspects of citrate synthase

Else, Anthony J.

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SOME ASPECTS

OF

CITRATE SYNTHASE

submitted by Anthony J. Else

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ABBREVIATIONS

Most of the abbreviations used in this thesis are those recommended in the Biochemical Society publication "Policy of the Journal and Instructions to Authors" (1985).

Non-standard Abbreviations

AcCoA: acetyl-coenzyme A

BSA: bovine serum albumin

CS: citrate synthase

cmc: critical micelle concentration

DCPIP: dichlorophenol-indophenol

DTNB: 5,5' dithiobis-(2-nitrobenzoic acid)

DNPH: 2,4-dinitrophenylhydrazine

E3: lipoamide dehydrogenase

GDH: glutamate dehydrogenase

GOGAT: glutamine-oxoglutarate aminotransferase

IDH: isocitrate dehydrogenase

LDH: lactate dehydrogenase

MDH: malate dehydrogenase

OA: oxaloacetate

palCoA: palmitoyl-coenzyme A

palthio: palmitoyl-thioglycollate

SSC: 150mM NaCl + 150mM Na citrate, pH 7.0

SSPE: 250mM NaH_2PO_4 buffer, pH 7.0, containing 150mM NaCl & 1mM EDTA

thy: thymine

TCA: trichoroacetic acid

TEA: triethanolamine

ET8: 20mM Tris-HCl buffer, pH8.0, containing 1mM EDTA

GET8: ET8 + 20%(v/v) glycerol

KET8: ET8 + 100mM KCl

TE8: 10mM Tris-HCl buffer, pH8.0, containing 1mM EDTA

STE: TE8 + 100mM NaCl

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CONTENTS

SUMMARY

1. The methods of assay of citrate synthase are reviewed. A discontinuous assay using 2,4-dinitrophenyl-hydrazine to measure oxaloacetate was developed which can be used in the presence of thiols.

2. The effects of palmitoyl-coenzyme A and related compounds on the citrate synthases of Escherichia coli, Bacillus megaterium, and pig heart were investigated. Palmitoyl-coenzyme A inhibited all of these citrate synthases, the Escherichia coli enzyme being the most sensitive. Palmitoyl- thioglycollate was also found to be an inhibitor of the citrate synthases; again, the Escherichia coli enzyme was the most sensitive. Breakage of the thioester bond of these compounds destroyed the inhibitory effects. Palmitate caused a 30% inhibition of Escherichia coli citrate synthase; it did not inhibit the citrate synthases of Bacillus megaterium or pig heart.

3. A model for predicting the patterns obtained on proteolysis of multisubunit enzymes is presented. The model is applied to the trypsinolysis of the citrate synthases of Escherichia coli, Bacillus megaterium, and pig heart. The resulting analysis suggests that the dimer is the basic active unit of citrate synthases.

4. An attempt to clone the citrate synthase gene from an Escherichia coli strain containing a mutant citrate synthase is described. A similar method was also used to attempt to clone the citrate synthase gene from Bacillus

subtilis.

A Bacillus subtilis mutant, CU1695, was found to lack citrate synthase, but it could not be used in the cloning experiments because of its complex growth requirements. Attempts were made to obtain a more suitable citrate synthase deficient mutant of Bacillus subtilis.

5. Wild-type Escherichia coli, when grown on glucose minimal medium, overproduced a compound that enabled the growth of a citrate synthase deficient Escherichia coli strain. Chromatographic analysis suggested that this compound was 2-oxoglutarate.

1 INTRODUCTION

1.1 The Citric Acid Cycle

1.1.1 Elucidation of the Citric Acid Cycle and the Citrate Synthase Reaction

The scheme of the terminal stages in the oxidation of carbohydrates by animal tissues was still in debate when Krebs & Johnson (1937) proposed the pathway that has become known as the citric acid cycle (or alternatively as the tricarboxylic acid or the Krebs cycle). Some of the reactions of the proposed cycle had been known to occur in muscle tissue since 1911 when Batelli & Stern (1911) demonstrated the rapid oxidation of citrate, succinate, fumarate, and malate in frog muscle. In 1936 the work of Martius & Knoop (1936) revealed the mechanism of the conversion of citrate to succinate, leaving the demonstration of the formation of citrate from oxaloacetate (OA) and pyruvate (Krebs & Johnson, 1937) to 'close' the cycle. At this time, the details of the formation of citrate were not known.

Novelli & Lipmann (1947) showed that coenzyme A (CoA), which had been discovered by Lipmann *et al.* (1947), was involved in citrate synthesis. Stern *et al.* (1950) showed that the reaction proceeded via the formation of 'active acetate' which, by the action of 'condensing enzyme', was combined with OA. Isolation and chemical analysis of 'active acetate' identified it as acetyl-coenzyme A (AcCoA) (Lynen & Reichert, 1951).

'Condensing enzyme', now known as citrate synthase (CS), catalyzes the reaction:

$$AcCoA + OA + H_2O \rightleftharpoons Citrate + CoASH + H^+$$

The citric acid cycle is illustrated in Fig. 1.1; it is now known to be the pathway for the final oxidation of all major foodstuffs in the vast majority of living organisms, and even in organisms that lack the cycle per se, parts or individual enzymes of the cycle can usually be found (e.g., in Trichomonads).

1.1.2 The Dual Role of the Citric Acid Cycle

Oxidation of AcCoA by the citric acid cycle produces CO_2 and reduced pyridine nucleotides; reoxidation of the nucleotides is coupled to energy production, i.e., the formation of ATP.

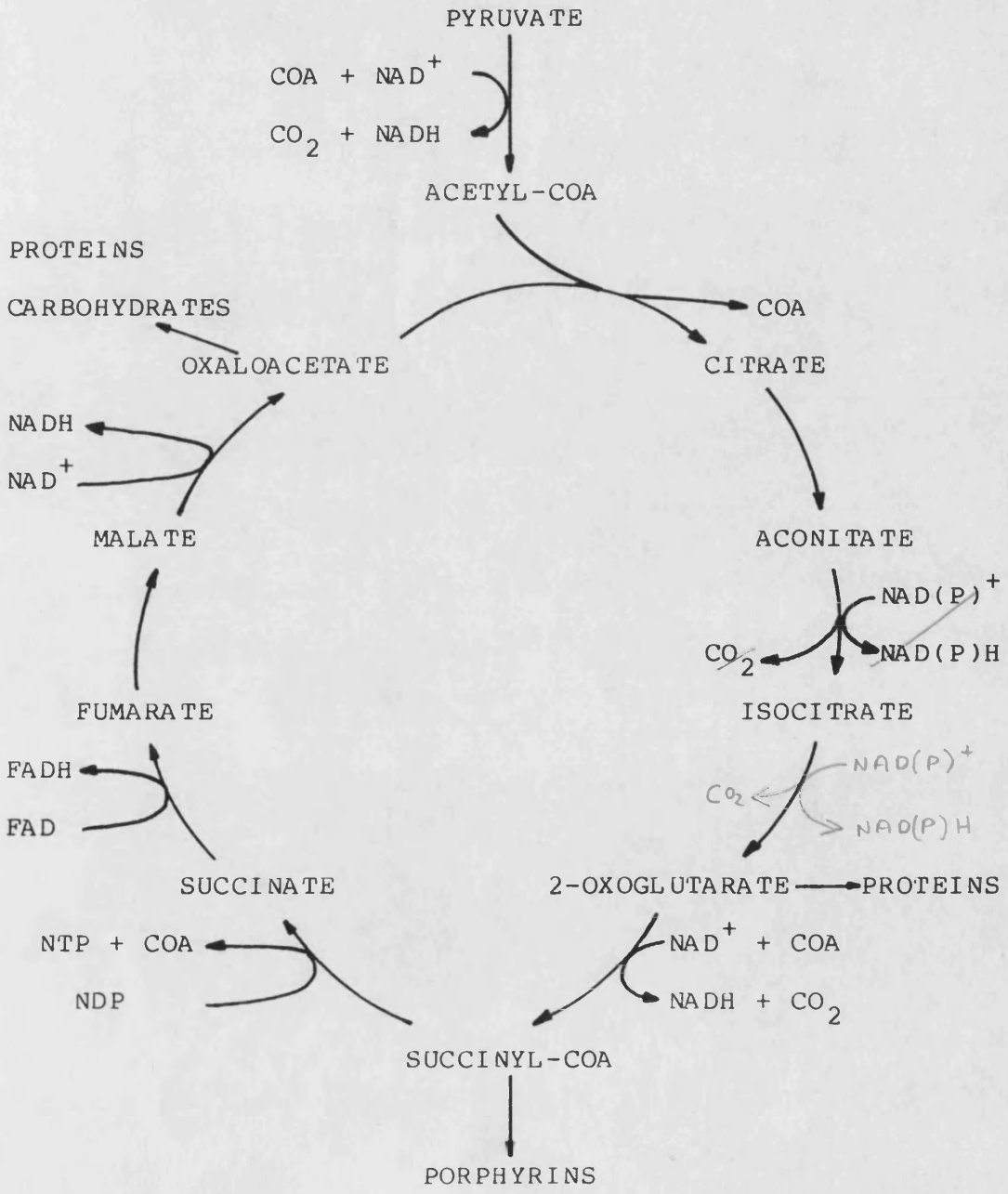
Krebs et al. (1952) suggested a second vital role for the cycle: the supply of biosynthetic precursors for cell components. Support for this second role was provided by tracer studies of Roberts et al. (1953). The dual role of the cycle is illustrated in Fig. 1.1.

1.1.3 The Citric Acid Cycle as the Train Station of Metabolism

Catabolism of all major foodstuffs produces citric acid cycle intermediates. Carbohydrates and fatty acids are catabolized to AcCoA which is fed into the cycle by CS. Alternatively, carbohydrates can be fed into the cycle at the stage of pyruvate, via pyruvate

Figure 1.1: The Citric Acid Cycle

The role of the cycle as a producer of energy (via reduction of pyridine nucleotides) and of cell constituents is illustrated.



carboxylase. Amino acids, derived from proteins, enter the cycle via pyruvate, AcCoA, 2-oxoglutarate, succinyl-CoA, fumarate, or OA depending on the amino acid in question.

Conversely, in anabolism, the citric acid cycle serves as a source of biosynthetic precursors for proteins, porphryins (from succinyl-CoA), fats (from citrate and/or AcCoA), and carbohydrates (from OA).

The cycle can be seen to serve as a central co-ordinating pathway. It is like a train station with arriving and departing molecules, carrying carbon chains and associated energy. Regulation of the cycle ensures that all of these arrive in the right place at the right time, and that they depart on time to their required destination. In this sense, it is more efficient than any train station hitherto constructed by Man. The reason for this is that, like all metabolic pathways, it is self-regulating; the regulation is a result of parts of the system acting on or referring to other parts of the system.

1.2 Citrate Synthase

1.2.1 The Key Role of Citrate Synthase in the Regulation of the Cycle

Krebs & Lowenstein (1960) deduced that the primary control of the rate at which the citric acid cycle operates must be exerted on the synthesis of citrate, a conclusion drawn from the facts that cycle intermediates do not normally accumulate and that

addition of such intermediates often leads to an increased oxygen uptake. CS catalyzes the reaction that is responsible for the major 'feeding in' of carbon into the cycle, and in a sense, it can therefore be considered the first enzyme of the cycle. As such it is a likely candidate for feedback inhibition. Such inhibition is observed with ATP or NADH and will be discussed in detail in 1.3.2.

Further evidence for the key regulatory role of CS was provided by McMinn & Ottaway (1976) who used a digital computer simulation to study the control of the citric acid cycle in rat heart. Applying the theory of Kacser & Burns (1973, 1979), they calculated the sensitivity coefficient of CS to be 0.835 (all others < 0.083) and concluded that control lay largely with this enzyme. The sensitivity coefficient of an enzyme is defined as the fractional change in flux through a pathway over the fractional change in the enzyme's activity. It has a theoretical upper limit of unity; the nearer to unity the greater that enzyme's influence on the pathway.

1.2.2 Extent of the Current Knowledge of Citrate Synthase

Considering the importance of CS in central metabolism, it is not suprising that it has been widely studied in a variety of organisms, and much information is now available. The enzyme has been purified from pig heart (Srere, 1969), pigeon breast and moth flight muscle (Ochoa et al., 1951), the Gram-negative bacterium E.coli

(Weitzman, 1969), the Gram-positive thermophile Bacillus stearothermophilus (Higa & Cazzulo, 1976), the Gram-positive Bacillus megaterium (Robinson et al., 1983a), and several other sources. The pig heart and the E.coli enzymes have been the most extensively studied. The complete amino acid sequence of the pig heart enzyme has been determined (Bloxham et al., 1981, 1982); the enzyme has been crystallized and analyzed by X-ray crystallography (Remington et al., 1982) revealing two different crystal forms, designated as open and closed; it has been suggested that these two crystal forms correspond to two catalytically distinct forms: hydrolase and ligase (Lohlein-Werhahn et al., 1983). They propose that pig heart CS is a hysteretic enzyme existing in two interconvertible forms which alternate during the catalytic cycle; binding of OA to the open (hydrolase) form generates the closed (ligase) form, and the consumption of OA with formation of the suspected intermediate, citryl-CoA, regenerates the open form.

The gene coding for E.coli CS has been cloned (Guest, 1981) and the base sequence determined (Hull et al., 1983; Ner et al., 1983). X-ray crystallography data is also available for E.coli CS, but at a low resolution (Rubin et al., 1983).

Further discussion of the properties of CS necessitates a review of the diversity in these properties from organism to organism, and the realization that there is not merely one type of CS, but a whole

family of similar but distinct types.

1.3 Diversity Amongst Citrate Synthases

1.3.1 Molecular Size

A marked dichotomy has been revealed in the molecular size of CSs. The CSs of eukaryotes and Gram-positive bacteria are dimers of relative molecular mass in the region of 100,000; the CSs of the Gram-negative bacteria are hexameric with relative molecular mass of approximately 250,000 (Weitzman & Dunmore, 1969a; Wu & Yang, 1970a; Singh *et al.*, 1970; Moriyama & Srere, 1971; Wiegand *et al.*, 1979; Tong & Duckworth, 1975; Higa & Cazzulo, 1976; Robinson *et al.*, 1983a).

1.3.2 Inhibition by Nucleotides

As discussed in 1.2.1, CS can be considered the first enzyme in a pathway leading to the production of ATP. Therefore, ATP is a likely candidate for an inhibitor of CS, such an inhibition providing an efficient feedback regulation of ATP production. Hathaway & Atkinson (1965) observed that CS from yeast could be inhibited *in vitro* by concentrations of ATP within the physiological range. Inhibition was competitive with respect to AcCoA, and ATP had its greatest effect when ADP and AMP concentrations were low. As a consequence of these and other observations, Atkinson (1968) developed the concept of energy charge, in which it is the relative rather than the absolute concentrations of the adenine nucleotides which are of metabolic significance. Though

the concept of energy charge has been somewhat discredited, the rationale for feedback inhibition of CS by ATP remains.

As well as yeast, ATP inhibition of CS has been reported in a wide variety of organisms (Weitzman & Danson, 1976). Doubt has however been cast upon the physiological significance of this inhibition in view of the finding that the inhibition was diminished by the addition of divalent cations such as Mg^{2+} (Kosicki & Lee, 1966; Lee & Kosicki, 1967). Also, *in situ* studies with yeast cells made permeable to substrates by treatment with toluene (Weitzman & Hewson, 1973) and with toluenized rat liver mitochondria (Matlib *et al.*, 1978) produced a diminished response of CS to ATP.

NADH can also be considered as an end product of the citric acid cycle, and so feedback inhibition of CS by NADH would be an efficient method of regulation. Weitzman (1966a,b) found that whilst ATP was a relatively poor inhibitor of *E.coli* CS, NADH was a powerful allosteric inhibitor. The CS of *Acinetobacter calcoaceticus*, another Gram-negative bacterium, was also found to be inhibited by NADH (Weitzman & Jones, 1968); in this case, the inhibition could be relieved by low concentrations of AMP. A survey of a number of bacterial species (Weitzman & Jones, 1968) placed the organisms investigated into two distinct groups: the enzyme from all the Gram-negative species tested was inhibited by NADH, but no such inhibition was observed with the

Gram-positve species. The Gram-negative bacteria could be further divided on the basis of the type of NADH inhibition: the strict aerobes displayed AMP reactivation, but no reactivation was observed with the facultative anaerobic organisms. A rationale for this second subdivision was proposed: facultative anaerobes can produce energy by fermentation, without recourse to the citric acid cycle, and they possess glycolytic enzymes that are sensitive to low levels of ADP and AMP; strict aerobes are dependent upon the citric acid cycle for their energy production and so require a low energy signal to modulate the cycle.

1.3.3 R and S Forms

The vast majority of CSs are si stereospecific. However, a few strictly anaerobic bacteria have re stereospecificity (Gottschalk & Barker, 1966, 1967; Stern & Bambers, 1966; Gottschalk & Dittbrenner, 1970).

1.4 Biosynthetic Controls

Nucleotide inhibition of CSs may be considered to be concerned with the energy requirements of the organism. As a consequence of the diversity in the physiology of organisms, there is also a diversity in the biosynthetic controls of CS.

1.4.1 Inhibition of Citrate Synthase from Gram-negative Facultative Anaerobes by 2-Oxoglutarate

Facultative anaerobes such as E.coli can satisfy their energy requirements by the fermentation of appropriate substrates, and this occurs under anaerobic

conditions and even under aerobic conditions with a suitable substrate such as glucose. In these circumstances, the citric acid cycle is not required to produce energy; a branched non-cyclic adaption of the cycle operates instead in order to provide the important biosynthetic intermediates, succinyl-CoA and 2-oxoglutarate (Amarasingham & Davis, 1965)(Fig. 1.2). Whilst the formation of succinyl-CoA does not depend upon CS, 2-oxoglutarate is the end product of a short linear pathway starting from CS. Weitzman & Dunmore (1969b) showed that the CSs of the Gram-negative facultative anaerobes examined were all inhibited by 2-oxoglutarate, but those of other bacteria and eukaryotes examined were unaffected. Further studies (Taylor, 1970; Tanaka & Hanson, 1975; Gottschalk & Dittbrenner, 1970) have revealed that the effect is not restricted to Gram-negative facultative anaerobes, but is a feature of organisms lacking 2-oxoglutarate dehydrogenase.

1.4.2 Inhibition of Citrate Synthase from Cyanobacteria by 2-Oxoglutarate and Succinyl-CoA

The Gram-negative cyanobacteria also lack 2-oxoglutarate dehydrogenase, and their CS is found to be inhibited by 2-oxoglutarate (Taylor, 1973), but not by NADH (Lucas & Weitzman, 1975). Moreover, succinyl-CoA is formed from the operation of the glyoxalate cycle (Fig. 1.3)(Pearce et al., 1969; Lucas, 1974); it is therefore an end product of a linear sequence from CS, and it has been confirmed as an inhibitor of cyanobacterial CS

Figure 1.2: The Split Cycle Mode of the Citric Acid Cycle

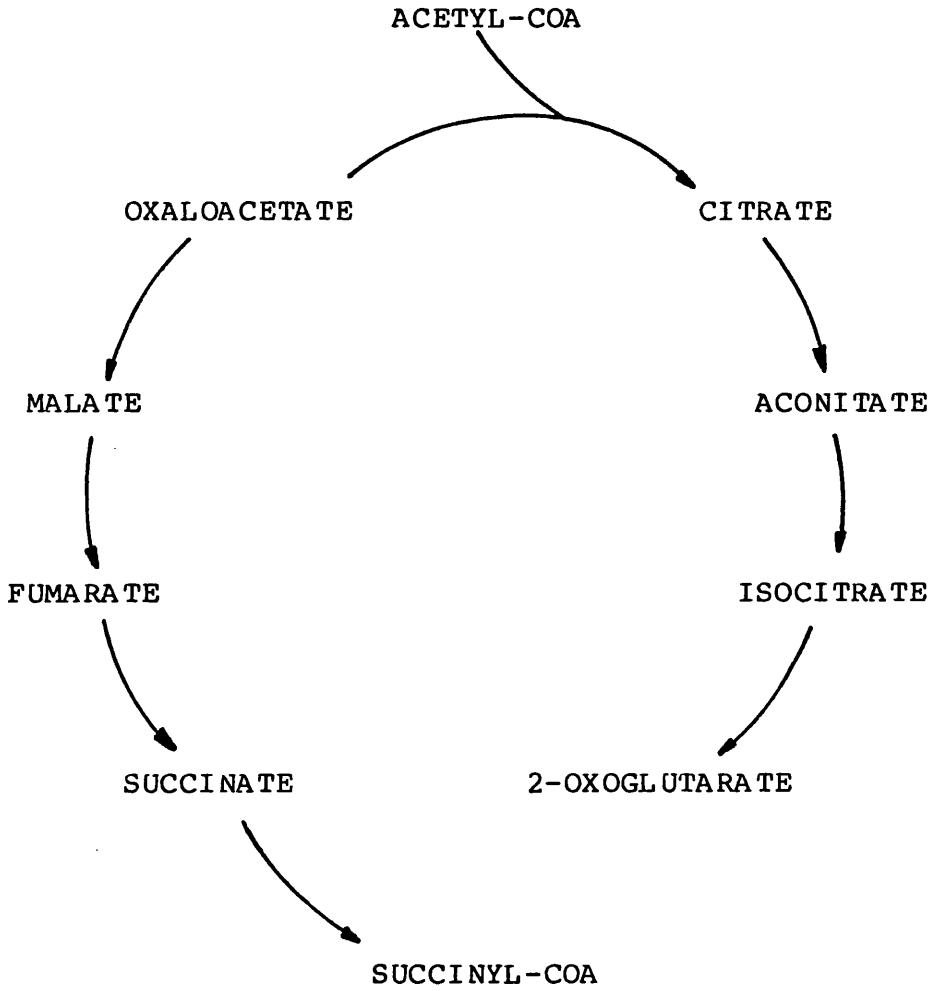
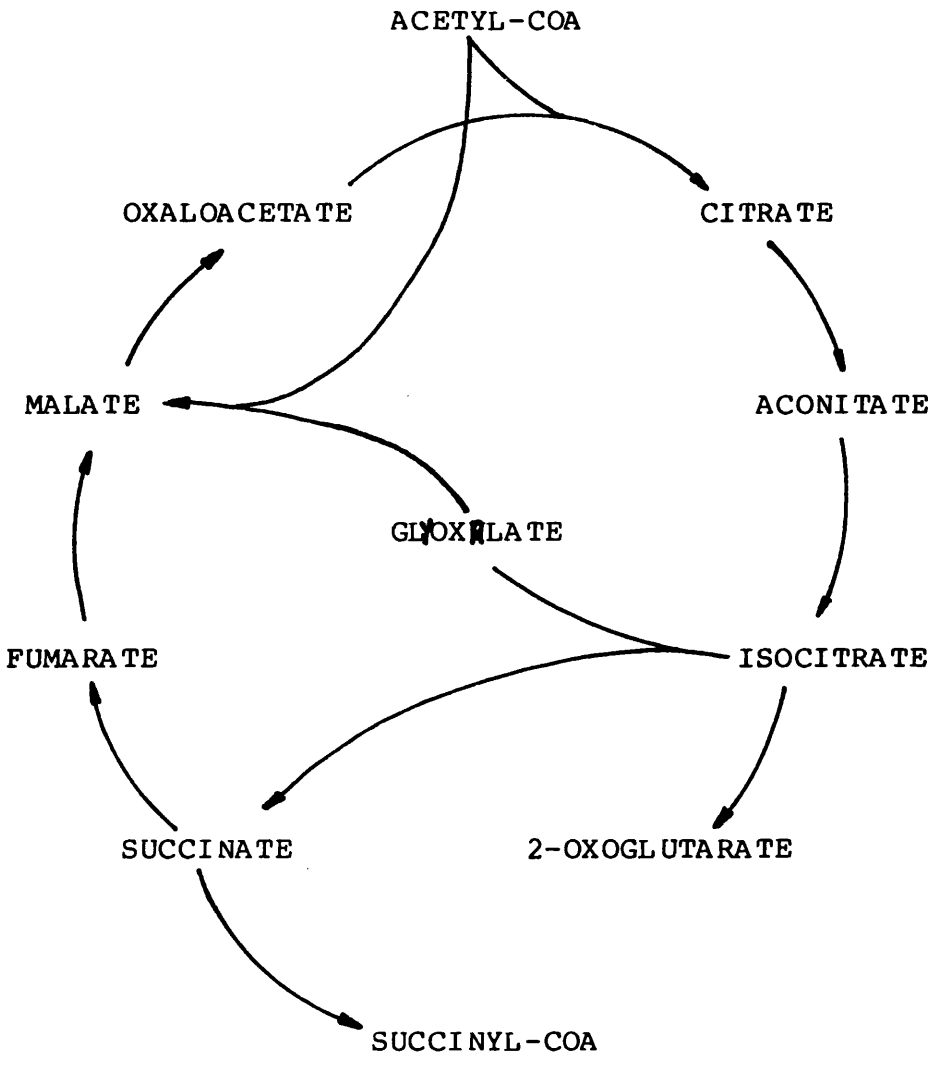


Figure 1.3: The Glyoxalate Cycle and Modified Citric Acid Cycle Operating in Cyanobacteria



Succinyl-CoA and 2-oxoglutarate act as end-product inhibitors of citrate synthase from cyanobacteria.

(Lucas & Weitzman, 1977) whereas it has no effect upon the CSs of E.coli or Acinetobacter calcoaceticus. The inhibition by succinyl-CoA is competitive with respect to AcCoA; the inhibition by 2-oxoglutarate is competitive with OA.

1.5 Inhibition of Citrate Synthase by Fatty Acyl-CoA

Mammalian CS is inhibited by fatty acyl-CoAs such as palmitoyl-CoA (palCoA) at low concentrations (around 5μM)(Wieland & Weiss, 1963; Tubbs, 1963). It was believed that this inhibition could play a part in regulation of citrate levels and therefore of the biosynthesis of fatty acids and of ketone bodies (ketogenesis). The physiological significance of the inhibition was questioned (Srere, 1965) because palCoA is an excellent detergent, and several aspects of the inhibition suggested that it was caused by a detergent-like action. More recently, however, by using analogues of palCoA, a specific binding of palCoA to pig heart CS has been demonstrated (Hsu & Powell, 1975; Caggiano & Powell, 1979).

In this work, a comparative study of the effects of palCoA and related compounds upon the CSs of pig heart, E.coli, and Bacillus megaterium was undertaken. The topic is therefore discussed in more detail in chapter 5.

1.6 Ionic Effects

CSs do not have a requirement for a specific ion, but the activity and regulation of the enzyme from a

variety of sources are modulated by monovalent and divalent ions.

1.6.1 Monovalent Cations

Increase of KCl concentration up to about 60mM increases the activity of several mammalian and plant CSs; inhibition is observed with higher concentrations (Eggerer et al., 1964; Poulsen & Sarkissian, 1971; Srere, 1971; Wu & Yang, 1970b). This effect is not unique to KCl, but is observed with a range of monovalent ions and is therefore probably due to changes in ionic strength. It results in changes in the affinity of the enzyme for AcCoA, V and Km for OA being unchanged (Wu & Yang, 1970b).

The reversible inhibition of pig heart CS by high concentrations of KCl is probably due to salt-induced conformational changes in the enzyme, but does not involve dissociation or complete unfolding of the subunits (Wu & Yang, 1970a & 1970b).

Activation by monovalent salts has also been demonstrated in both Gram-positive (Johnson & Hanson, 1974; Flechtner & Hanson, 1970) and Gram-negative bacteria (Faloona & Srere, 1969; Flechtner & Hanson, 1970; Wright & Sanwal, 1971; Johnson & Hanson, 1974; Massarini & Cazzulo, 1974), and here again, it is the affinity for AcCoA that is affected.

KCl has also been shown to desensitize bacterial CSs to their inhibitors: the E.coli CS was first reported to be desensitized to NADH inhibition

(Weitzman , 1966b) and subsequently to the inhibition by 2-oxoglutarate (Wright et al., 1967) and palCoA (Srere & Whissen, 1967). Loss of NADH inhibition cannot be totally accounted for by changes in affinity for AcCoA, as increasing the NADH concentration does not overcome the desensitization. This behaviour has been reported for other bacteria which are inhibited by NADH, but where the NADH inhibition can be relieved by AMP the desensitization can be overcome by higher concentrations of NADH. The significance of this is discussed in 1.7.

1.6.2 Divalent Cations

High concentrations (approximately 100 x [AcCoA]) of Mn^{2+}, Mg^{2+}, or Ca^{2+} inhibit pig heart CS (Kosicki & Lee, 1966; Lee & Kosicki, 1967); the suggestion is that this is due to chelate formation between the divalent cation and the polyphosphate chain of AcCoA. High concentrations of Mg^{2+} (approximately 100mM) inhibit several bacterial CSs (Faloona & Srere, 1969; Flechtner & Hanson, 1970). However, low concentrations (approximately 5mM) are stimulatory, possibly due to the formation of an OA-Mg chelate complex which would make the α-carbon more electrophilic and thus facilitate the nucleophilic attack by AcCoA; Mg may therefore be physiologically important for the activity of CS (Weitzman & Danson, 1976).

1.7 Significance of the Diversity; the Allosteric Behaviour of Some Citrate Synthases

As discussed above, the CSs of a variety of

organisms fall into various groups that correspond remarkably with the taxonomic classification of the organisms. In some cases, the diversity in regulation can be rationalized in terms of the different requirements of the organisms (e.g., biosynthetic controls, AMP reactivation of NADH inhibition). However, no explanation has been provided for the 'great divide' between the 'small', ATP-inhibited CSs of eukaryotes and the Gram-positive bacteria, and the 'large', NADH-inhibited CSs of the Gram-negative bacteria. The same division exists when one looks at succinate thiokinase: eukaryotes and Gram-positive bacteria have 'small', dimeric enzymes, whilst the Gram-negative bacteria have 'large' tetrameric enzymes. There is also some evidence that pyruvate dehydrogenase complexes follow a similar pattern. Weitzman (1981) has reviewed the diversity of citric acid cycle enzymes.

Returning to CS, it is significant that the large size is associated with NADH inhibition, for these CSs display the characteristics of both the 'K' and 'V' systems for allosteric regulatory enzymes (Monod et al., 1965). The two groups of NADH-sensitive CSs display different kinds of kinetic behaviour. With facultative anaerobes (e.g., E.coli), a hyperbolic dependence of activity with respect to NADH concentration is observed, but the dependence of activity with respect to AcCoA concentration is sigmoidal, indicative of positive co-operativity between the subunits (Weitzman, 1966a;

Faloona & Srere, 1969; Wright & Sanwal, 1971). In the absence of salts, between pH 8.0 & 9.5, E.coli CS exists as a mixture of species of different relative molecular masses (60,000, 240,000, & 480,000) which are in dynamic equilibrium (Wright & Sanwal, 1971; Danson & Weitzman, 1973). In the presence of KCl, only the 240,000 species is present and the co-operativity is not observed. The salt-desensitization of the enzyme to inhibition by its allosteric effectors must therefore result in the absence of the subunit interactions. Turning to the second group, we find a sigmoidal dependence of activity with respect to NADH and hyperbolic kinetics with AcCoA (Weitzman, 1967; Eidels & Preiss, 1970; Flechtner & Hanson, 1970; Boriss & Ohmann, 1972; Johnson & Hanson, 1974; Kleber & Tauchert, 1974; Massarini et al., 1976; Higa et al., 1978). The dependence of reactivation on the concentration of AMP is also sigmoidal. Electron microscopy studies of Acinetobacter calcoaceticus CS show that KCl increases the diameter of the enzyme molecule and reverses the size changes induced by NADH (Rowe & Weitzman, 1969).

The inhibition by ATP, which is competitive with respect to AcCoA, is dependent upon the affinity of the enzyme for that substrate. A plot of Km_{AcCoA} against Ki_{ATP} for the CSs from a wide variety of organisms shows a very strong correlation between these parameters (Weitzman, Harford, & Danson, unpublished results). The CSs of Gram-negative bacteria which are poorly inhibited

by ATP have a relatively high Km_{AcCoA}. ATP thus inhibits because it is an analogue of AcCoA, as discussed in 1.3.2, the in vivo significance of ATP inhibition is questionable.

1.8 Some Additions and Exceptions to the General Pattern of Citrate Synthase Diversity

1.8.1 Some Unusual Citrate Synthases

The overwhelming majority of CSs studied have followed the patterns discussed, but a few exceptions have been discovered. Most notable perhaps is the case of Acetobacter xylinum, a Gram-negative bacterium which has a large but NADH-insensitive CS (Swissa & Benziman, 1976).

Phibbs & Winkler (1982) reported that the CS from the obligate intracellular parasite Rickettsia prowazekii, a Gram-negative bacterium, has a relative molecular mass of 62,000 and is inhibited by ATP.

The CS from the gills of the fish, Antimora rostrata, when observed under normal laboratory conditions existed as a large type with a relative molecular mass of 270,000. But when it was subjected to conditions similar to those of the habitat of the fish, namely 200-250 atmospheres pressure, the enzyme depolymerized into a more active enzyme of relative molecular mass 100,000 (Hochachka, 1975).

1.8.2 Pseudomonad Citrate Synthases

A mutant of Pseudomonas aeruginosa has recently been found to contain both a large and a small CS

(Solomon & Weitzman, 1983). As a result of this finding, a survey of a range of Pseudomonad species was undertaken which revealed that some contain both large and small CSs, whereas others contain either the large or the small form (Mitchell & Weitzman, 1986).

1.8.3 Archaebacterial Citrate Synthases

As well as eukaryotes and prokaryotes, a third category of organisms is now recognized: the Archaebacteria (Woese, 1981). They are characteristically found in environments of extreme conditions, and it is thought that they might be closely related to some of the earliest forms of life on earth. In the few species that have been studied so far, small, NADH-insensitive CSs have been found (Danson et al., 1985). In the case of the halophiles, Cazzulo (1973) suggested that as inhibition of CS by NADH is overcome by high salt concentrations, the adaption to high salinity has resulted in the loss of this mode of regulation which has been rendered ineffective by these conditions. With the recognition of halophiles as archaebacteria, Cazzulo's argument should perhaps be put the other way round.

1.8.4 Mutant Citrate Synthases

In studies with E.coli (Harford & Weitzman, 1978; Danson et al., 1979a), a CS deficient strain (CS^-) was allowed to revert spontaneously; the revertant strains studied were found to have one of three different types of CS. Apart from apparently wild-type revertants, a large NADH-insensitive enzyme (resembling that of

Acetobacter xylinum) and also a small, NADH-insensitive, ATP-sensitive enzyme (resembling the Gram-positive/eukaryotic type) were found. In the case of the last type, it appears that relatively minor genetic changes have resulted in a drastic change in the properties of the enzyme: this mutant has crossed the 'great divide'.

1.9 Enzyme Diversity; Structure-function Relationships; the Significance of Studies of Mutant Citrate Synthases

Enzyme diversity is studied for three major reasons:-

1. In order to provide information about isoenzymes and differential enzyme distribution which can have important uses in diagnostics.
2. From an evolutionary viewpoint, to show patterns of relatedness of organisms.
3. In order to obtain information about the structure-function relationships of organisms.

The second and especially the third reasons are the most important in terms of this study.

As Szekely (1980) has pointed out, the structure and function of any chemical substance are related whether it takes part in a simple chemical reacton or in a complex biological process. The question is however of more interest in a macromolecular system, where the relationship can be disguised by the complexity of the molecules involved. It is then not easy to decide which parts of the molecule are responsible for its

various observed properties. Also, as indicated by information theory, a degree of redundancy can be tolerated (or may even be necessary) in complex systems, and so not all parts of a macromolecule may be significantly involved in function.

From organism to organism, the physiological demands upon the citric acid cycle will vary, as we have seen, and this variation is manifested in a diversity of the regulation of CS. The major difference is that between the small non-allosteric ATP-inhibited CSs and the large allosteric NADH-inhibited enzymes. Here we have exemplified a structure-function relationship in a complex macromolecular, biological system: ATP inhibits by virtue of its competitive binding to the AcCoA site and so does not depend upon co-operative effects of a multisubunit enzyme; on the other hand, NADH inhibits by an allosteric mechanism which is dependent upon multisubunit interactions that only occur in the large, hexameric CSs.

This relationship is at a level of quaternary protein structure (i.e., subunit interactions); to probe in more depth, to see what modifications of primary, secondary, and tertiary structure are important to the differences in quaternary structure, more detailed comparisons of the different types of CSs are required. This is one of the major purposes of current studies of CSs.

The diversity observed in naturally occurring

CSs is an obvious focus for comparative structure-function studies. However, evolutionary divergence has almost certainly introduced substantial variation in amino acid sequences that are unconnected with the key structural and functional diversity. However, with the mutant E.coli CSs, we have the diversity of structure and function produced by minor genetic changes, i.e., with minimal changes in amino acid sequence. The study of these mutants is therefore a 'cleaner' approach to this problem, since the redundant parts of the molecule should be nearly identical in all the mutant enzymes.

1.10 Aims of the Work Described

This thesis describes a variety of different approaches to the study of CSs, all aimed at probing the structure-function relationships that have been discussed.

There were two initial aims. The first aim was to compare Bacillus CS with the E.coli and pig heart enzymes, which have already been well studied. The E.coli CS is a large eubacterial CS, the pig heart enzyme is a small eukaryotic CS. Bacillus CS stands in the middle of these, in that it is a small eubacterial CS, and it is therefore important in any comparative study of CSs. Bacillus megaterium was used as the source of Bacillus CS, but the microbiological and genetic studies were carried out with Bacillus subtilis. The CSs of these two species are indistinguishable in terms of kinetic and

molecular properties. The studies with palCoA thus served two purposes: they were part of a comparative study of CSs, and they were also aimed at trying to determine whether or not palCoA is important for the *in vivo* function of CS. The second aim was to clone the gene for the small mutant *E.coli* CS. This was important because the mutant enzyme is produced in small quantities and it probably has a lower specific activity than the wild type enzyme; it is consequentely difficult to purify. Cloning may permit amplification of the levels of the mutant enzyme in the cell and thus facilitate purification. The mutant enzyme could of course also be directly studied at the level of DNA. It was hoped that in cloning the mutant CS, a more general procedure for the cloning of CS genes could be worked out.

2. MATERIALS

2.1 Organisms

The major bacterial strains used in this work are listed in Tables 2a & 2b. The other bacteria used were Acinetobacter calcoaceticus 4B, the pseudomonads: Ps.acidovorans D1870, Ps.aeruginosa 1978, PAC1, & PAC514, Ps.alcaligenes D123, Ps.chlororaphis D302, Ps.diminuta D1032, Ps.iodinum, Ps.maltophilia D144, Ps.putida, Ps.saccharophila D1021, Ps.stutzeri, Ps.testosteroni D1047, all from the culture collection of Professor P.D.J. Weitzman (University of Bath), and the archaebacteria: Thermoplasma acidophilum DSM1728, Sulpholobus acidocaldarius DSM639, & Natranococcus occultus NCMB2192.

2.2 Chemicals

Tryptone, yeast extract, and agar were from Difco Labs., Detroit, U.S.A.; nutrient broth and nutrient agar were from Oxoid Ltd., London, U.K.; CoA, OA, NADH, NADPH, and $NADP^+$ were from Boehringer, Mannheim, F.R.G.; acrylamide, ammonium persulphate, cysteine hydrochloride, DL-lipoamide, 2-mercaptoethanol, sodium dodecyl sulphate, and N,N,N',N'-tetramethylethylene diamine were from BDH Chemicals Ltd., Poole, U.K.; protamine sulphate (salmon roe) was from Koch-Light, Colnbrook, U.K.; bromophenol blue and PEG6000 were from Fisons, Loughborough, U.K.; CsCl was from BRL, Cambs., U.K.; Sephadex G-50, Sephadex

Table 2: Bacterial Strains

(A) Genotype and Source

Organism	Genotype	Source
E.coli		
K12	Wild-type	PDJW[a]
K11r3	Hfr,*pps*,*met*,*thy*	PDJW
W620	*thi1*,*pyrD36*,*gltA6*,*galK30*,*rpsl129*	Guest[b]
DB1002	*thi1*,*pyrD36*,*galK30*,*rpsL129*	Bloxham[c]
HB101	F^-,*hsds20*(r_B^-,m_B^-),*recA13*,*ara14* *proA2*,*lacY1*,*galK2*,*rpsL20*(str^r) *xyl5*,*mtl1*,*supE44*,λ^-	Bloxham
B.megaterium		
D101	Wild-type	PDJW
B.subtilis		
168	*trp*	Sargent[d]
CU1695	*trp*,*ilvA*,*citD*,*citK*,*glt*,SPB	Sargent
CU1323	*trp*,*metB5*,*citK1*, *ilvB*,*kanA1*,*gltA2*	Sargent
MS160	*trp*,*met*,*citK*, *ilvB*,*kanA1*	Sargent
UTB600	*trp*,*gltB*,*leuA*,*metB*	Sargent

a From the culture collection of Prof. P.D.J.Weitzman, The University of Bath

b A gift of Prof. J.R.Guest, The University of Sheffield

c A gift of Dr. D.Bloxham, The University of Southampton

d A gift of Dr. M.Sargent, MRC, Mill Hill, London

Table 2: Bacterial Strains

(B) Growth Requirements and Other Comments

Organism	Special Growth Requirements	Other Comments
E.coli		
K12	none	
K11r3	met & thy	gltA revertant "small" CS
W620	glu, uracil, & thiamin	CS^-
DB1002	uracil & thiamin	contains WT *E.coli* CS gene cloned in pBR322
HB101	leu & pro	
B.megaterium		
D101	none	
B.subtilis		
168	trp	
CU1695	numerous	derived from 168 by large deletion
CU1323	trp,met,ile,val, & glu	
MS160	trp,met,ile, & val	glt^+ derivative of CU1323
UTB600	trp,glu,leu, & met	

G-200, and DEAE-Sephacel were from Pharmacia; Matrex Gel Red A was from Amicon, Lexington, U.S.A.; [α-^{32}P]dCTP was from Amersham, Bucks., U.K.; all other fine chemicals were from Sigma, Poole, U.K.

2.3 Enzymes

Yeast triose phosphate isomerase was from Boehringer, Mannheim, F.R.G.; BSA (Pentax fraction V) was from Miles Scientific, Slough, U.K.; bovine pancreatic trypsin, soyabean trypsin inhibitor, pig heart citrate synthase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, egg white lysozyme, RNAse A, pronase, and calf intestinal alkaline phosphatase were from Sigma, Poole, U.K.; T4 DNA ligase, ECoRl, HindIII, and the nick-translation kit were from BRL.

E.coli pyruvate dehydrogenase complex was purified by the method of Danson *et al.* (1979b).

Rat brain palCoA deacylase was kindly provided by Dr. R.V. Brunt (University of Bath).

2.4 Special Preparation of Reagents

DL-dihydrolipoamide was prepared by the reduction of DL-lipoamide with $NaBH_4$ (Reed *et al.*, 1958).

Phenol was redistilled before use. The redistilled phenol can be stored at -20^{o}C. When required, it was allowed to warm to room temperature, and then melted at 68^{o}C. 8-hydroxyquinoline was then added to a

final concentration of 0.1%(w/v). The phenol was then extracted with an equal volume of 1M Tris-HCl pH 8.0 followed by 0.1M Tris-HCl pH 8.0 containing 0.2%(v/v) 2-mercaptoethanol. This phenol solution was then kept at 4°C under the equilibration buffer until required.

Chloroform refers to a mixture of chloroform and isoamyl alcohol (24:1 v/v). This mixture is stable and was stored in closed bottles at room temperature.

Denatured salmon sperm DNA was prepared by passing a 10mg/ml solution of the DNA in water several times through a 19-gauge hypodermic needle. The DNA was then boiled for 10 min and stored at -20°C. Just before use, it was heated for 5 min in a boiling-water bath and then chilled quickly in ice.

3. METHODS

3.1 Maintenance and Growth of Organisms

All bacterial strains were maintained on nutrient agar plates and subcultured regularly (trimonthly for most strains, bimonthly for *Bacillus subtilis* mutant strains).

Growth in liquid culture was at 37°C with shaking.

3.2 Culture Media

For routine growth of organisms, either L-broth (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl - Lennox, 1955) or nutrient broth were used.

Defined media for growth of *E.coli* strains were based on the basal salts medium of Ashworth and Kornberg (1966): 50mM Na/K PO_4 buffer pH 7.2 with 50mM NH_4Cl, 0.18mM $CaCl_2$, 0.33mM $MgSO_4$, 0.018mM $MnSO_4$, & 0.014mM $FeSO_4$ with the appropriate carbon/energy source and any growth requirements (see Table 2b).

Defined media for *Bacillus* species were based on Spizizen's minimal medium (Anagnostopoulos & Spizizen, 1961): 50mM Na/K PO_4 buffer pH 7.4 with 1mM $MgSO_4$, 15mM $(NH_4)_2SO_4$, 0.45 M $MnSO_4$, & 3.5 M $FeSO_4$ with the appropriate carbon/energy source and any growth requirements (see Table 2b).

For solid media, 15g/l of agar were added.

When required, antibiotics were added to the autoclaved media after cooling to 60°C. Ampicillin was

added to give a concentration of 25mg/l. Tetracycline was added to give a concentration of 12mg/l. Plates with antibiotics were stored at 4°C until required (within 1 week). Media and plates with tetracycline were stored in the dark.

3.3 Gram Staining

Gram staining was performed as described by Gillies & Dodds (1973). A loopful of the bacteria from an 18h-old colony on agar was spread on a microscope slide in a drop of 0.85% saline. The slide was dried in air and the film of material fixed by passing the slide three times through a bunsen flame. The slide was flooded with methyl violet, left for 5min and then rinsed with Gram's iodine. After a further 2min, the slide was drained and the cells decolourised by exposure to acetone for 5s before washing in water. Neutral red was applied as a counterstain, and after 30s, the slide was rinsed with water and blotted dry.

3.4 Enzyme Assays

All assays were carried out at 25°C.

In all cases, a unit of enzyme activity is defined as that amount that can catalyze the conversion of 1μmol of substrate per min.

3.4.1 Citrate Synthase (EC 4.1.3.7)

A review of the methods of assay of CS is given in chapter 4, and a new assay using 2,4-dinitrophenylhydrazine (DNPH) to measure the disappearance of OA is also presented there.

For routine assays of CS, the continuous, spectrophotometric method employing Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used (Srere et al., 1963). Unless otherwise stated, the assay mixture contained 0.15mM AcCoA, 0.2mM OA, & 0.1mM DTNB in the appropriate buffer: for pig heart CS and Bacillus CS, 20mM Tris-HCl pH 8.0 + 1mM EDTA (ET8); for E.coli CS, either ET8 or ET8 + 0.1M KCl (KET8).

The increase in absorbance at 412nm due to the production of the yellow-coloured thio-nitrobenzoate anion was monitored (e_{412} 13,600 $l.mol^{-1}.cm^{-1}$).

3.4.2 Malate Dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase (MDH) was assayed by following the decrease in absorbance at 340nm due to the oxidation of NADH (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2mM OA & 0.2mM NADH in ET8 buffer. Any NADH dehydrogenase was accounted for.

3.4.3 Lactate Dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase (LDH) was assayed by following the decrease in absorbance at 340nm due to the oxidation of NADH (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2mM pyruvate & 0.2mM NADH in ET8 buffer. Any NADH dehydrogenase was accounted for.

3.4.4 Isocitrate Dehydrogenase (EC 1.1.1.42)

Isocitrate dehydrogenase (IDH) was assayed by following the increase in absorbance at 340nm due to the reduction of $NADP^+$ (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 4mM isocitrate, 0.2mM $NADP^+$, & 10mM

$MgCl_2$ in ET8 buffer.

3.4.5 NAD(P)H Dehydrogenase (EC 1.6.99.1 & 1.6.99.3)

NADH & NADPH dehydrogenases were assayed by following the decrease in absorbance of a 0.2mM solution of NADH or NADPH in ET8 buffer at 340nm (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$).

3.4.6 Glutamate Dehydrogenase (EC 1.4.1.2 & 1.4.1.4)

Glutamate dehydrogenase (GDH) was assayed by following the decrease in absorbance at 340nm due to the oxidation of either NADH or NADPH (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 2mM 2-oxoglutarate, 0.2mM NAD(P)H, & 10mM NH_4Cl in ET8 buffer. NAD(P)H dehydrogenases were accounted for.

3.4.7 Glutamine-oxoglutarate Aminotransferase (EC 2.6.1.15) Glutamine + 2-oxoglutarate + NAD(P)H + H^+ → 2 glutamate + NAD(P)$^+$

Glutamine-oxoglutarate aminotransferase (GOGAT) was assayed by following the decrease in absorbance at 340nm due to the oxidation of either NADH or NADPH (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 4mM glutamine, 2mM 2-oxoglutarate, 0.2mM NAD(P)H, & 10mM $MgCl_2$ in ET8 buffer. NAD(P)H dehydrogenase and GDH were accounted for.

3.4.8 Acetyl-coenzyme A Deacylase

AcCoA deacylase was assayed by following the increase in absorbance at 412nm due to the formation of the thio-nitrobenzoate anion (e_{412} 13,600 $l.mol^{-1}.cm^{-1}$) from DTNB. The assay mixture contained 0.15mM AcCoA & 0.1mM DTNB in the appropriate buffer (ET8 or KET8).

3.4.9 Palmitoyl-coenzyme A Deacylase

PalCoA deacylase was assayed by following the increase in absorbance at 412nm due to the formation of the thio-nitrobenzoate anion (e_{412} 13,600 $l.mol^{-1}.cm^{-1}$) from DTNB. The assay mixture contained between 1 and 10 M palCoA, and 0.1mM DTNB in ET8.

3.4.10 Dihydrolipoamide dehydrogenase (EC 1.6.4.3)

Dihydrolipoamide dehydrogenase (E3) was assayed by following the increase in absorbance at 340nm due to the reduction of NAD^+ (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 0.4mM dihydrolipoamide and 2.5 mM NAD^+ in 50mM KPO_4 buffer, pH 7.0

3.4.11 2-Oxoglutarate Dehydrogenase (EC 1.2.4.2)

2-Oxoglutarate dehydrogenase (2OGDH) was assayed by following the increase in absorbance at 340nm due to the reduction of NAD^+ (e_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 2mM oxoglutarate, 2.5mM NAD^+, 0.2mM thiamine pyrophosphate, 0.13mM CoA, 2.6mM cysteine-HCl, and 1mM $MgCl_2$ in 50mM KPO_4 buffer, pH 8.0.

3.5 Estimation of Proteins

For most purposes, protein concentration was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

UV absorbances at 280nm and 260nm were used to determine recoveries of protein following column chromatography.

For rapid estimation of protein concentration in crude preparations, or where interfering substances

such as CoA prevented estimation by UV absorbances, the Bio-rad (Bradford, 1976) micro-protein assay was employed, using lysozyme as standard.

3.6 Preparation of Acetyl-coenzyme A

AcCoA was prepared by the method of Stadtman (1957). 10mg of CoA were dissolved in 1ml of double-deionized water and the solution cooled in ice. 0.2ml of 1M $KHCO_3$ were added to bring the solution to pH7.5. 0.2ml of freshly diluted 0.1M acetic anhydride were added and the solution left on ice for at least 10min. This should give a solution of approximately 7mM AcCoA. Acetylation was tested with DTNB.

3.7 Estimation of Acetyl-coenzyme A

The concentration of AcCoA was determined by using the standard assay for CS (3.4.1) with pig heart CS. OA and DTNB were in approximately three-fold excess of AcCoA, and the reaction was allowed to go to completion. The concentration of AcCoA was then calculated from the increase in absorbance at 412nm.

3.8 Estimation of Oxaloacetate

Estimation of OA is discussed in chapter 4. It was routinely estimated by the same method as for AcCoA (3.7), but with AcCoA and DTNB in excess of OA.

3.9 Preparation of Palmitoyl-thioglycollate and Palmitoyl-coenzyme A

PalCoA was prepared by the two-step method of Chase & Tubbs (1972). The first step involves the preparation of palmitoyl thioglycollate (palthio); palCoA

is then prepared by treating CoA with an excess of palthio. 20mmol (5g) of palmitic acid was dissolved in 15ml of trifluoroacetic acid and 18mmol (2.5ml) of trifluoroacetic anhydride added. The mixture was left at room temperature for 1h. 20mmol (1.4ml) of thioglycollic acid (redistilled and stored at -20°C; White,1960) were added and the mixture left for a further 30min. A few ml of water were then added to break down anhydrides, and the mixture evaporated to dryness in vacuo. Palthio was then recrystallized from hexane: the mixture was dissolved in hexane by warming to 60°C, filtered hot to remove unwanted material, and filtered cold to collect the crystals. 530µmol (175mg) of the palthio crystals were dissolved in 3.6ml of a 1:1(v/v) mixture of 2-methyl-propan-2-ol and 0.5M $NaHCO_3$ at 35°C. 50mg of CoA, dissolved in 1ml of the same mixture, were added to the palthio solution to give a monophasic solution at pH 8.0-8.5 (pH is critical). This mixture was left at 35°C for 45min, and then cooled on ice. 20%(v/v) perchloric acid was added dropwise to bring the solution to pH 3. The tertiary butanol was evaporated by flushing with N_2, and the solution adjusted to pH 1-2 with 20%(v/v) perchloric acid. The mixture was washed six times with equal volumes of diethyl ether; this causes the bottom phase to clear and a spongy precipitate to be deposited at the interface. The ether was removed with N_2 and the precipitate collected by centrifugation (2,000g, 4°C for 10min). The precipitate was washed twice with ice-cold

2%(v/v) perchloric acid, and collected by centrifugation as before. The precipitate was resuspended in 1ml of 25mM Na/K PO_4 buffer, pH7.4; this gives a solution of approximately pH4. The palCoA is best kept in this acid solution and frozen.

3.10 Estimation of Palmitoyl-coenzyme A

The concentration of palCoA, prepared as 3.9, was determined by two different methods:-

1. By measuring the absorbance at 260nm (e_{260} 16.4 x 10^6 $l.mol^{-1}.cm^{-1}$)

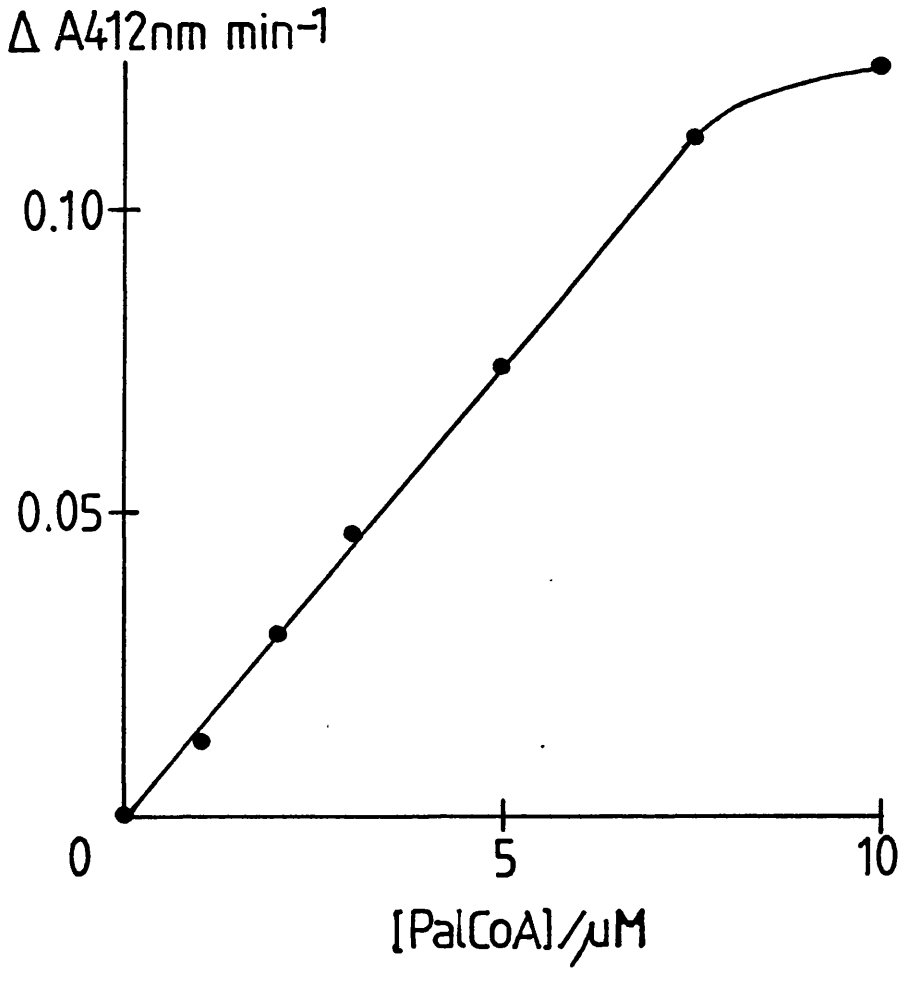
2. By comparison with palCoA standards in a palCoA deacylase assay (3.4.9) (see Fig.3.1) using a preparation of rat brain palCoA deacylase.

Values obtained by method 2 were approximately 1/3rd of those from method 1. The values quoted are those obtained from method 1. Preparations were usually found to be approximately 5mM.

3.11 Purity of Palmitoyl-coenzyme A

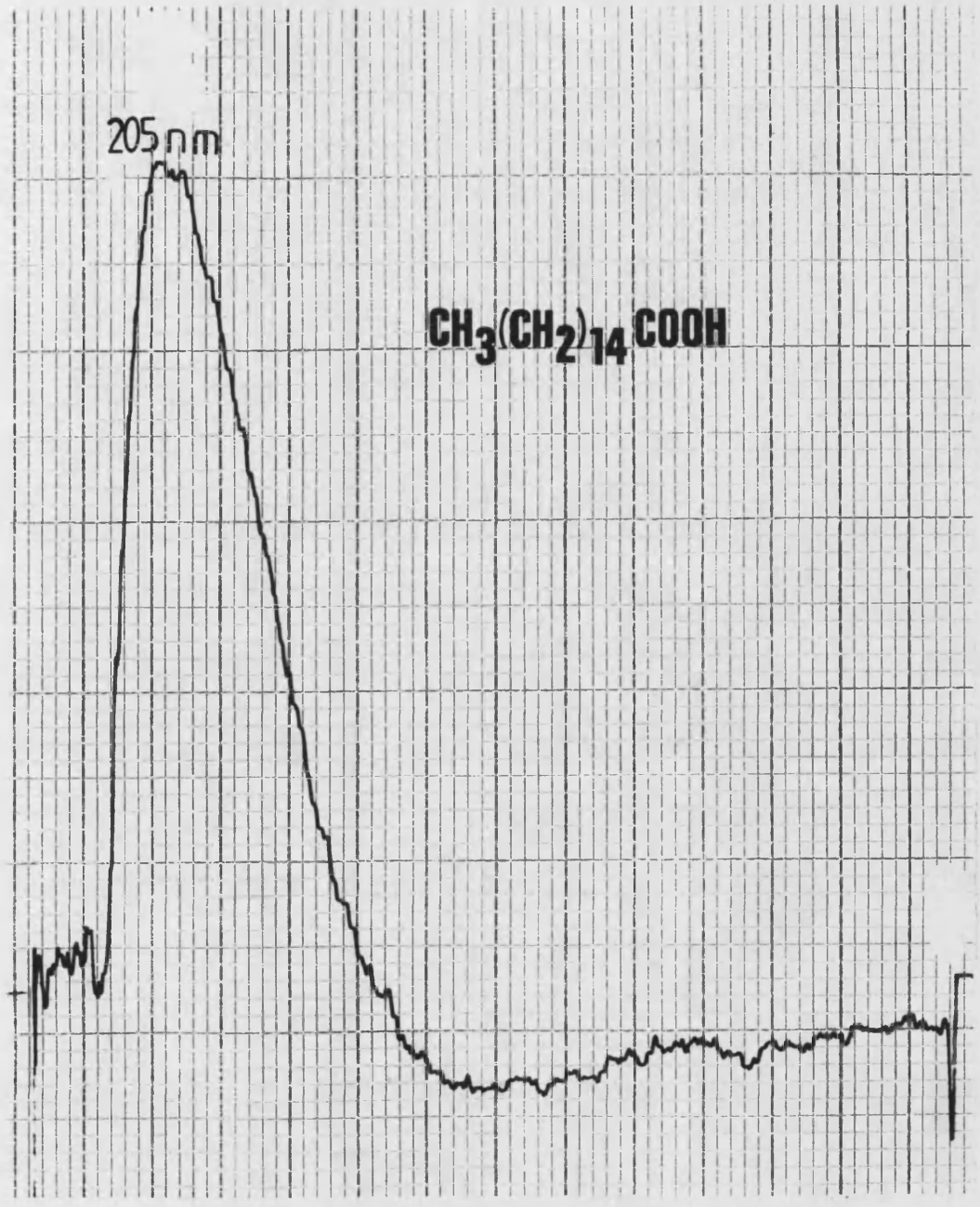
The purity was determined on the basis of adenine absorbance at 260nm and the absorbance at 232nm (e_{260} 16.4 x 10^6 $l.mol^{-1}.cm^{-1}$; e_{232} 9.4 x 10^6 $l.mol^{-1}.cm^{-1}$). Greater absorbance at 232nm may indicate the presence of other thio esters which have a high absorbance at this wavelength. The mean purity of five preparations was 91% $\pm$ 4%(sem). Figures 3.2-3.5 show spectral scans of palmitic acid, palthio, commercially obtained palCoA, and a preparation of palCoA made as described above.

Figure 3.1: Standard Curve for the Determination of Palmitoyl-coenzyme A Concentration by Rat Brain Palmitoyl-coenzyme A Deacylase



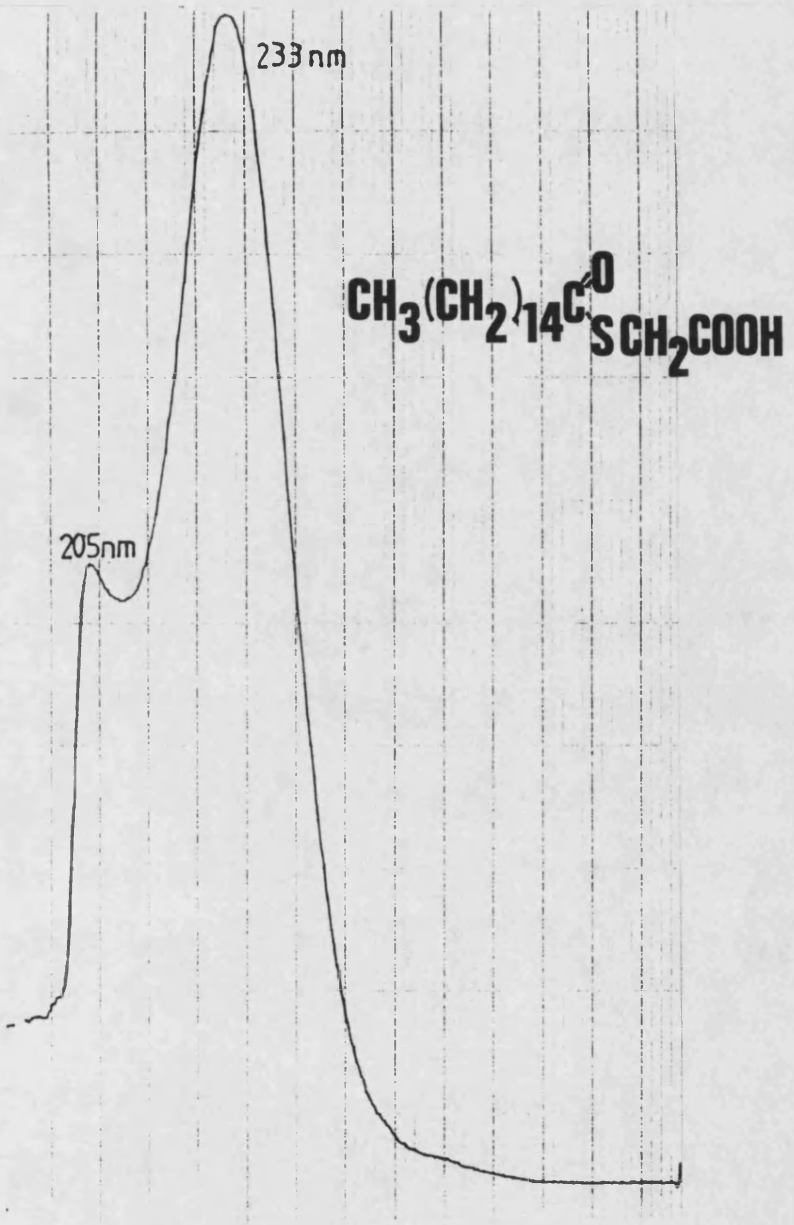
The assays were carried out as described in 3.49, using 10μl of a 100x dilution of a preparation of rat brain deacylase.

Figure 3.2: Spectrophotometric Scan of Palmitic Acid



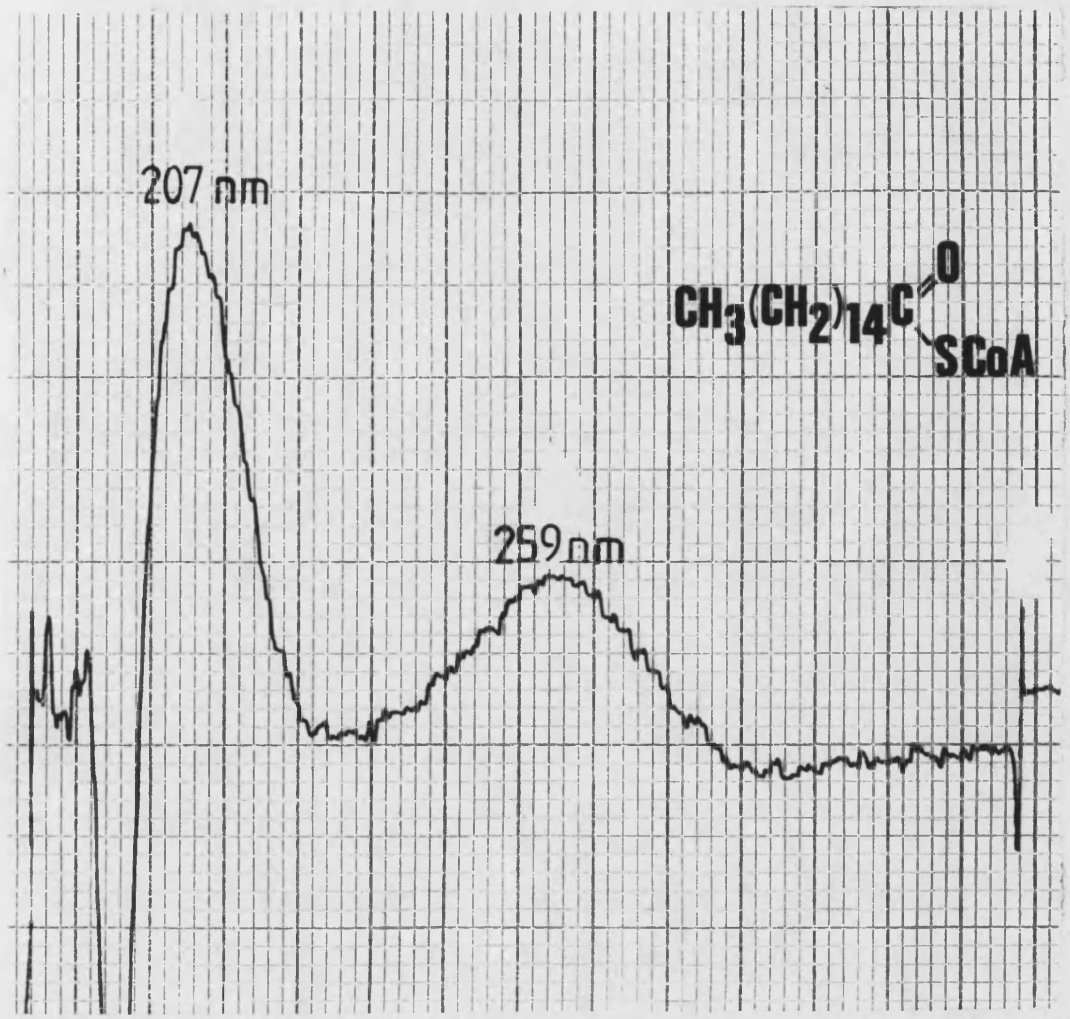
The scan was of a 2mM solution of palmitic acid in 95%(v/v) ethanol, scanning from 200nm to 322nm (left to right). On the ordinate, 2.5cm correspond to 0.02 absorbance units.

Figure 3.3: Spectrophotometric Scan of Palmitoyl-thioglycollate



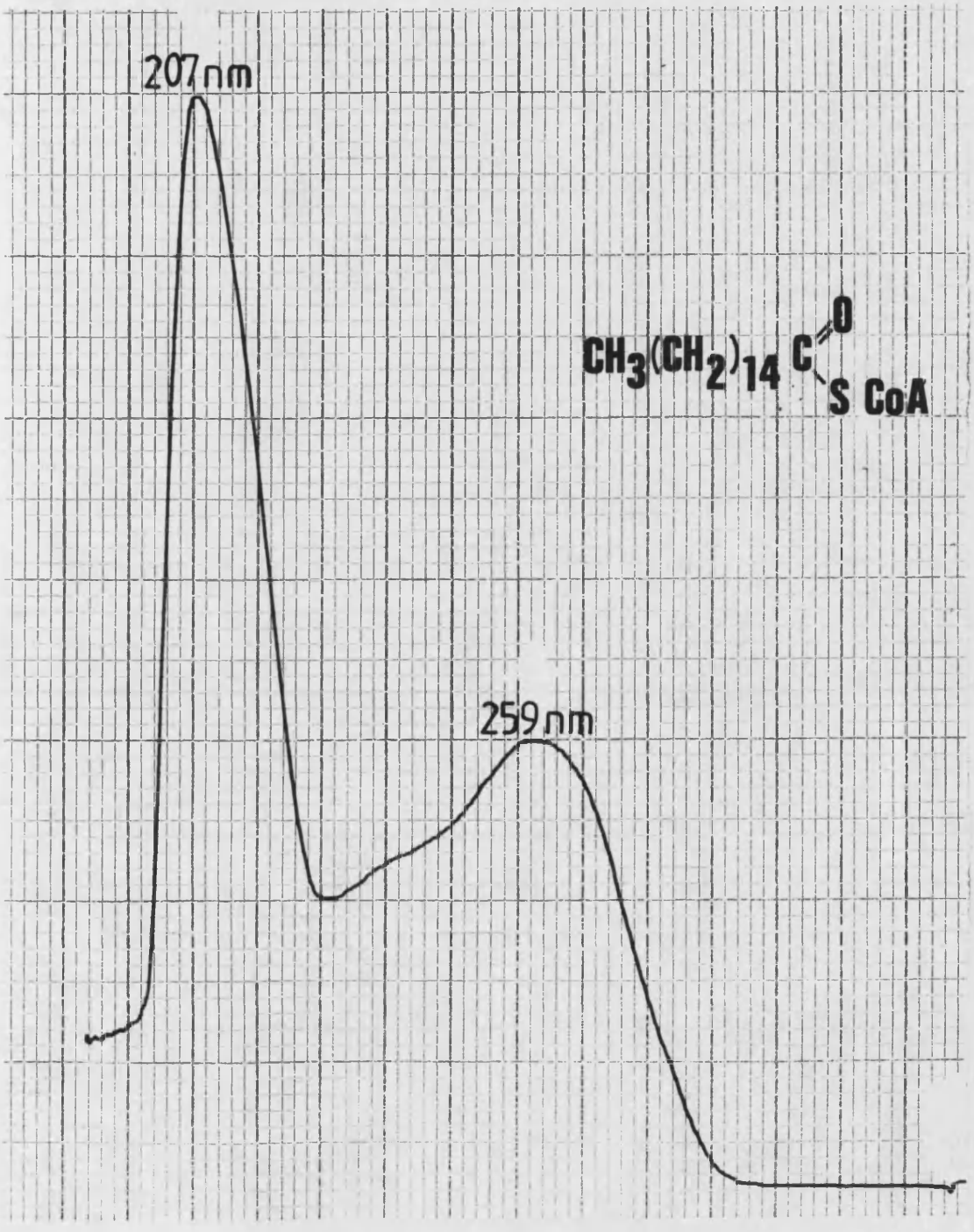
The scan was of a 0.6mM solution of palthio in 95%(v/v) ethanol, scanning from 200nm to 322nm (left to right). On the ordinate, 1.6cm correspond to 0.1 absorbance units.

Figure 3.4: Spectrophotometric Scan of Commercial Palmitoyl-coenzyme A



The scan was of a 1.3μM solution of commercial (Sigma) palCoA in 95%(v/v) ethanol, scanning from 200nm to 322nm (left to right). On the ordinate, 2.5cm correspond to 0.02 absorbance units.

Figure 3.5: Spectrophotometric Scan of Palmitoyl-coenzyme A Prepared as Described in 3.9



The scan was of a 30μM solution of palCoA in 95%(v/v) ethanol, scanning from 200nm to 322nm (left to right). On the ordinate, 2.5cm correspond to 0.2 absorbance units.

3.12 Polyacrylamide Gel Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out according to Davis (1964) using 10% polyacrylamide gels prepared in 12cm x 0.6cm glass tubes. The resolving gel buffer was 0.375M Tris-HCl pH 8.9, the stacking gel buffer was 0.125M Tris-HCl pH 6.8, and the reservoir buffer was 0.025M Tris, 0.19M glycine pH 8.3. Polymerisation of the acrylamide-bisacrylamide was accomplished by using ammonium persulphate as an initiator, and N,N,N',N'-tetramethylethylenediamine (TEMED) as a catalyst. Approximately 10µg of protein were applied per gel in 30µl of reservoir buffer also containing 2mM EDTA, 10%(w/v) sucrose, and 0.001%(w/v) bromophenol blue. The sample was allowed to enter the gel at 0.5mA per gel, and the current was then increased to 2mA per gel; the electrophoresis was stopped after the dye had travelled approximately 10cm. The gels were then removed from the tubes by forcing distilled water between the gel and the tube with a syringe. Protein was visualised by staining with Coomassie Blue as described below.

3.13 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out according to the procedure of Weber & Osborn (1969), using 10% gels prepared in 12cm x 0.6cm glass tubes. The buffer used for both the gels and the

reservoir was 0.1M $NaPO_4$ pH 6.7 with 0.1%(w/v) SDS. Polymerisation of the acrylamide-bisacrylamide was accomplished by using ammonium persulphate and TEMED. 10µg samples, dried in a vacuum desiccator, were dissolved in 30µl 0.1M $NaPO_4$ buffer pH 6.7, 1%(w/v) SDS, 10%(v/v) glycerol, 0.14M 2-mercaptoethanol, and 0.001%(w/v) bromophenol blue; they were then heated in a boiling water-bath for 2min before loading onto the gels. Electrophoresis was carried out at 8mA per gel; the electrophoresis was stopped after the dye had travelled approximately 10cm. The gels were removed from the tubes and protein visualised in the same way as for non-denaturing PAGE.

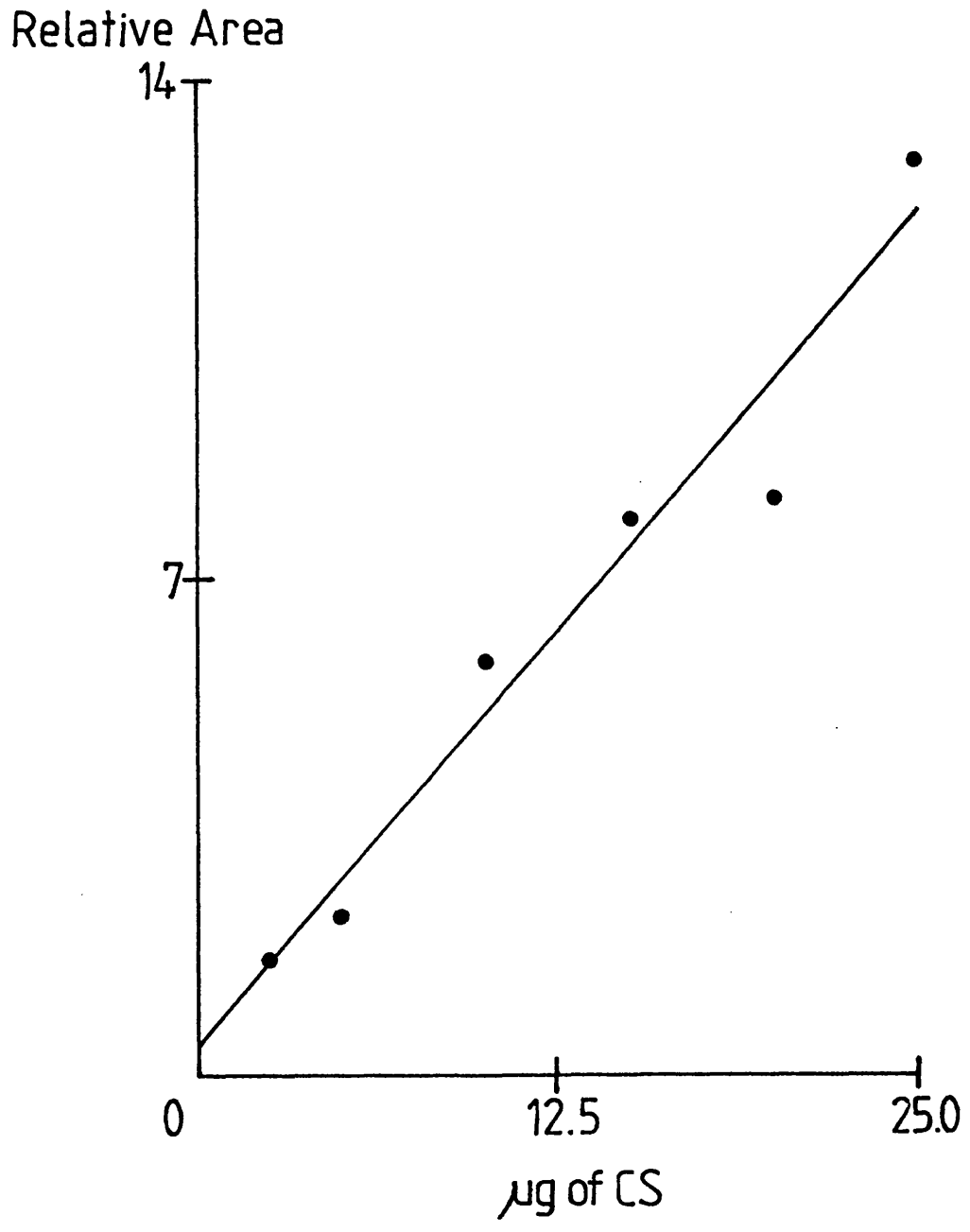
3.14 Staining of Polyacrylamide Gels with Coomassie Blue

Both SDS and non-denaturing polyacrylamide gels were treated in the same way.

After removing the gels from the glass tubes, fixing and staining were carried out simultaneously in a methanol:acetic acid: water mixture (5:1:5) with 1g/l Coomassie Brilliant Blue R, at 34°C for a minimum of 1h. The gels were then destained in a methanol:acetic acid:water mixture (2:3:35).

With SDS-PAGE, protein in the gels was quantified by using a gel scanner (ISCO model 1310) with an integrator (LDC/Milton Roy C1-10). The integrated area of the peaks obtained was linear with respect to the amount of CS added up to at least 25µg (Fig.3.6 shows the linearity of area with B.megaterium CS; similar figures

Figure 3.6: Integrated Area of a Gel Scan v Amount of Bacillus megaterium Citrate Synthase Added



Between 0 and 25µg of B.megaterium CS were loaded on a 10%SDS-polyacrylamide gel. Electrophoresis and staining were as described in 3.13 and 3.14 respectively.

were obtained with E.coli CS and with pig heart CS).

3.15 Harvesting of Cells and Preparation of Cell Extracts

Cells were harvested either by means of an Alpha-Laval Separator LAB 102 B-05 in the clarification mode (for large scale preparations, i.e., >1 l) or by centrifugation (11,000g at 4°C for 15min). The cells were resuspended in the appropriate buffer at a ratio of 1g of cells to 2ml buffer. With 10ml or more of resuspended cells, the cells were disrupted by passing the suspension twice through a pre-cooled French press at a pressure of 62MPa (9,000lb.f/in^2). With less than 10ml of resuspended cells, the cells were disrupted by sonication (Ultrasonics 180Watt sonicator) with a 5mm probe at a power of 50W for 1min in 15s bursts with cooling. In both cases, cell debris was removed by centrifugation (13,000g at 4°C for 30min).

3.16 Protamine Sulphate Precipitation

Nucleic acid was removed from cell extracts by precipitation with protamine sulphate. A 1%(w/v) aqueous solution was added dropwise to the cell extract (1mg protamine sulphate to 10mg protein). The extract, kept on ice, was stirred gently during the addition and left stirring for a further 30min after all the protamine sulphate had been added. The precipitate of nucleic acid was then removed by centrifugation (20,000g at 4°C for 30min).

3.17 Gel Filtration Chromatography

A column (35cm x 1.5cm) of Sephadex G-200 (swollen by heating for 5h in a boiling water-bath) was used to determine the approximate size of CSs (i.e., large or small type) in bacterial extracts.

The buffer used was the same as that in which the cell extract had been prepared. The chromatography was carried out at a flow rate of 10ml/h; 3ml fractions were collected.

Before application, the sample was filtered through a glass-fibre filter and approximately 0.1g sucrose added per ml to increase the density of the sample. 5µl of rabbit muscle LDH were also added to serve as a marker to distinguish between large and small CSs.

3.18 Purification of Citrate Synthase from Escherichia coli DB1002

CS from E.coli DB1002 was purified to homogeneity, as judged by 10% SDS-PAGE, by a 5-step procedure. A summary of the purification is presented in Table 3.1.

E.coli DB1002 was grown in L-broth for 18h. 9l of culture were used yielding 30g of cells from which 4g of protein were extracted by the French press. Following treatment with protamine sulphate, the extract was applied to a column (30cm x 3cm) of DEAE-Sephacel, equilibrated in ET8. The CS was eluted with a linear gradient of 0 to 0.5M NaCl in 500ml buffer. The flow rate

TABLE 3.1: Purification of E.coli DB1002 Citrate Synthase

	Volume (ml)	Total CS(U)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)
French Press extract	35	19355	3990	4.85	100
Protamine sulphate	62	18042	2108	8.56	93
DEAE-Sephacel	36	10800	389	27.78	56
FPLC	21	9900	206	48.06	51
FPLC rerun (2ml from 1st FPLC run)	13	1023	13.3	76.90	55*

The purification was carried out as described in 3.18.
Enzyme activity was measured in KET8 buffer throughout.

* Recalculated on the basis of the whole preparation

was 15ml/h, and 3ml fractions were collected. The enzyme was recovered in fractions containing approximately 0.3M NaCl. Fractions containing CS with specific activity greater than 20U/mg were pooled, diluted 10 times in 20mM triethanolamine (TEA) pH 7.3 with 1mM EDTA, and applied to a Pharmacia Mono Q ion-exchange column (FPLC system). Under these conditions, the CS was bound to the column; it was eluted by a linear gradient of 0 to 0.3M NaCl in 30ml of 20mM TEA pH 7.3 with 1mM EDTA. The flow rate was 1ml/min, and 1ml fractions were collected. Several runs were necessary in order to process all the material. Fractions with a specific activity greater than 45U/mg were pooled. 2ml of the pooled material was taken and diluted 10 times in 20mM TEA pH 7.3 with 1mM EDTA, and then 250µl aliquots were rerun on the Mono Q column under conditions identical with the first run. The material recovered from this second run had a specific activity of 77U/mg and gave a single band on 10% SDS-PAGE and three bands on 10% PAGE in the absence of SDS (pure E.coli CS is known to give 3 bands on PAGE under these conditions, see 1.7). The specific activity obtained is higher than that obtained for E.coli DB1002 CS (45U/mg) (Bloxham et al., 1983) and that obtained for the plasmid-encoded CS from E.coli JA200/pLC26-17 (63U/mg) (Robinson et al., 1983b) and is also higher than the specific activity of E.coli K12 CS (45U/mg) purified by the method of Weitzman (1969). The latter describes a 542-fold purification of the crude extract; the purification of E.coli DB1002 CS

by Bloxham et al. (1983) was 14-fold. The difference between latter result and the purification obtained here (16-fold of the crude extract) is probably explained by the difference in final specific activities obtained.

3.19 Purification of Bacillus megaterium Citrate Synthase

CS was purified from B.megaterium strain D101 by the method of Robinson et al. (1983a). A summary of the purification is presented in Table 3.2.

B.megaterium was grown in L-broth for 18h. 80g of cells were harvested from 20l of culture and were resuspended in GET8 containing 0.15mM phenylmethanesulphonyl fluoride (to inhibit serine protease activity) and 25mg/ml of lysozyme. The suspension was stirred continuously on ice for 2h. The cells were then lyzed by the French press and nucleic acid removed by protamine sulphate precipitation. The extract was applied to a column (30cm x 3cm) of DEAE-Sephacel equilibrated in GET8, and the CS eluted with a linear gradient of 0 to 0.5M KCl in 500ml of the same buffer. The increase in the total amount of CS after this step may be due to the introduction of KCl into the buffer rather than the elimination of an inhibitor. Fractions (5ml) were collected at a flow rate of 15ml/h. Two runs were needed in order to process all the material. Fractions with a specific activity greater than 0.25U/mg were pooled and dialyzed at $4^{o}C$ for 3h against 2l of GET8 containing 50mM KCl. The dialysate was applied to a column (25cm x 1.2cm) of Matrex Gel Red A that had

TABLE 3.2: Purification of Bacillus megaterium Citrate Synthase

	Volume (ml)	Total CS(U)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)
French Press extract	385	635	28875	0.02	100
Protamine sulphate	363	620	26136	0.02	98
DEAE-Sephacel	202	11	4241	0.27	182
1st dialysis	186	1033	3121	0.33	163
Red Gel Salt elution	83	578	115	5.03	91
2nd dialysis	80	559	119	4.70	88
Red Gel OA & CoA elution	13	507	6.1	84	80

The purification was carried out by the method of Robinson et al. (1983) as described in 3.19. Enzyme activity was measured in ET8 buffer throughout.

been equilibrated with GET8 containing 50mM KCl. A gradient of 0 to 0.5M NaCl was applied in 100ml of the same buffer, and 1ml fractions were collected at a flow rate of 10ml/h. Fractions with a specific activity greater than 4.8U/mg were pooled and dialyzed as before. This dialysate was then applied to a column (8cm x 0.8cm) of Matrex Gel Red A that had been equilibrated in GET8 containing 50mM KCl. CS was eluted by using this buffer containing 0.1mM CoA and 0.1mM OA; 1ml fractions were collected at a flow rate of 10ml/h. Two runs of both Matrex Gel Red A columns were required to process all the material.

Matrex Gel Red A can in theory be regenerated and reused many times, but it was found that the quality of the separations and the recoveries were decreased markedly after about 4 or 5 regenerations.

The final product had a specific activity of 84U/mg which is approximately 4 times that quoted by Robinson et al. (1983a), but is only about twice that obtained in some preparations by the same workers (Robinson, personal communication). It gave a single band upon both 10% SDS-PAGE and 10% PAGE in the absence of SDS.

3.20 General Procedure for Trypsinolysis

CS and trypsin were mixed together to give a CS/trypsin weight ratio of 100:1, and the reaction mixture was incubated at 25^{o}C. Proteolysis was monitored both by loss of catalytic activity and by loss of native

subunit upon SDS-PAGE. At recorded times, 60µl of the reaction mixture were taken and added to 100µl of ET8 containing 0.5µg soyabean trypsin inhibitor to stop further proteolysis. For measurements of catalytic activity, 10µl of this "stopped" solution were diluted by the addition of 240µl ET8 and 20µl of this diluted solution were sufficient for assay of CS. For SDS-PAGE, 15µl of the "stopped" solution were used.

Pig heart CS is not proteolysed by trypsin in the absence of pal-CoA. Trypsinolysis of pig heart CS was therefore carried out by including either 60 or 120µM pal-CoA in the reaction mixture.
Trypsinolysis of B.megaterium CS was studied in the presence and in the absence of 120µM pal-CoA, but with E.coli CS, which is more sensitive to pal-CoA (see 5.2.1), trypsinolysis was carried out only in the absence of pal-CoA.

For E.coli and pig heart CSs, all solutions were made in ET8; for B.megaterium CS, which is unstable in the absence of glycerol, GET8 was used exclusively.

3.21 Paper Chromatography of Organic Acids

Paper chromatography was perfomed by the method of Buch et al. (1952) using a 2-phase solvent system - 1-pentanol:5M aq. formic acid (1:1). 25µl of the standards (15mg/ml in distilled water) and 50µl of the Dowex (50 X8, H^+ form) treated samples were applied to a 20 x 20 cm sheet of Whatman No. 1 paper. The tank was equilibrated with the solvent system for at least 2h before the

chromatography. The paper was allowed to make contact with the upper, organic phase only. The spots were visualised after the chromatography by 0.04% (w/v) bromophenol blue in 95% (v/v) ethanol.

3.22 Thin-layer Chromatography of 2,4-Dinitrophenylhydrazine Derivatives

100µl of sample or standard (15mg/ml) were added to 200µl of 2%(w/v) DNPH in 1M HCl. Aldehydes and ketones produce a strongly yellow-coloured derivative. 200µl of ethyl acetate were then added. After vigorous shaking, two phases separated on standing, the coloured derivative being taken up by the upper organic phase. This upper phase was then used for the thin-layer chromatography.

25µl of the DNPH derivatives in ethyl acetate were spotted onto a TLC plate (10cm x 5cm); the solvent system used was butanol:acetic acid:water (3:1:1). Plates were run until the solvent front just reached the top; they were allowed to dry in air in a fume-cupboard. No development of the spots is necessary; the chromatography can be observed throughout.

3.23 Preparation of DNA from Escherichia coli Strains

Cells were harvested from 30ml of an 18h culture in L-broth. They were resuspended in 4ml of 50mM Tris-HCl pH 8.0, containing 25%(w/v) sucrose. Lysozyme (0.6ml of a 10mg/ml solution in 10mM Tris-HCl pH 8.0, 1mM EDTA (TE8)) was added, and the suspension incubated at 30°C for 1h with occasional mixing. 1.2ml of 0.5M EDTA and 0.7ml of predigested (30°C for 30min) pronase

(10mg/ml in TE8) were added, and the incubation at 30°C continued for 5min. SDS (3.6ml of 3.3%(w/v) in TE8) was added, and the solution mixed gently, but without vortexing. After incubation at 37°C for 2h, it was shaken with 6ml of phenol for 10min followed by the addition of 6ml of chloroform and shaking for 5min. The phases were separated by centrifugation in a 50ml tube in a bench centrifuge (5,000g at 4°C for 10min). The upper, aqueous phase was transferred to a fresh tube, and the shaking with phenol and with chloroform repeated. The aqueous phase was separated as before, transferred to a fresh tube, and RNAse (90°C for 10min to eliminate DNAse activity) added to give 40µg/ml. It was incubated at 37°C for 1h. NaCl (0.2 volumes of 0.5M) and PEG 6000 (0.5 volumes of 30%(w/v)) were added with gentle mixing, and the solution left standing overnight at 4°C. The precipitated DNA was collected by centrifugation (5,000g at 4°C for 10min). The pellet was gently dissolved in 5ml of TE8 containing 0.1M NaCl, and 0.5ml of 3M sodium acetate and 14ml of ethanol added (where unspecified, absolute ethanol is intended). The solution was mixed and left overnight at -20°C. The DNA was sedimented by centrifugation (22,000g at 4°C for 20min); the pellet was washed twice in ice-cold ethanol, dried in a vacuum desiccator for 3min, dissolved in sterile TE8, and stored at -20°C.

3.24 Preparation of DNA from Bacillus subtilis

The cells were grown to log phase in 100ml of

L-broth. They were harvested by centrifugation (5,000g at 4°C for 10min) and resuspended in 5ml of 10mM Tris-HCl buffer pH 7.5, containing 10mM NaCl & 0.1mM EDTA. Lysozyme was added to give a final concentration of 200µg/ml, and the suspension incubated at 45°C for 10min. Lauryl sarcosinate was added to give a 1%(w/v) solution, EDTA to give a final concentration of 50mM, and predigested (30°C for 30min) pronase to give a final concentration of 1mg/ml; the incubation at 45°C was continued for a further 20min. The solution was extracted with an equal volume of a 1:1 mixture of phenol and chloroform at room temperature for 30min with occasional vigorous shaking. The phases were separated by centrifugation (5,000g at 4°C for 10min), the upper aqueous phase taken, and the DNA precipitated from this phase by 2 volumes of ethanol containing 0.3M potassium acetate; the solution was left at -20°C for 30min to allow the DNA to precipitate. The DNA was collected by centrifugation (22,000g at 4°C for 20min) and dissolved in 10mM Tris-HCl buffer pH 7.5, containing 10mM NaCl & 0.1mM EDTA. The precipitation with ethanol and potassium acetate was repeated twice. After the last precipitation and resuspension, the DNA suspension was dialyzed overnight against the same buffer and then stored in aliquots at -20°C until required.

3.25 Preparation of Plasmid DNA

Cells from 200ml of culture were suspended in 2ml of 50mM Tris-HCl pH 8.0 containing 25% (w/v)

sucrose. 0.3ml of a lysozyme solution (10mg/ml in 50mM Tris-HCl pH 8.0) were added and the suspension gently swirled on ice for 5min. EDTA (1.2ml of a 0.25M solution) was added slowly and the suspension swirled on ice for a further 10min. 2.4ml of lysis solution (50mM Tris-HCl pH 8.0 containing 60mM EDTA and 2% (v/v) Triton) were added slowly. Chromosomal DNA was removed by centrifugation at 25,000g for 45min. The volume of the supernatant was adjusted to 6ml and 6g of CsCl and 0.6ml of ethidium bromide (10mg/ml) were added. The solution was centrifuged (82,000g at 23°C for 1h) and the pellet and floating material were discarded. The supernatant was recentrifuged (180,000g at 23°C overnight), and the plasmid band removed with a hypodermic needle. The ethidium bromide was removed by 6 extractions with CsCl-saturated isopropanol, the solution was dialyzed against several changes of TE8, and the DNA was precipitated with ethanol (3.32). It was redissolved in a small volume of TE8 and stored at -20°C.

3.26 Rapid Isolation of Plasmids

When plasmids were required for analysis rather than for cloning, a rapid isolation method was used.

Cells from 1.5ml of an 18h culture in L-broth were collected by centrifugation in a microfuge (12,000g for 1min). The supernatant was discarded, and after briefly respinning to remove liquid from the walls of the tube, the remaining medium was removed by aspiration. The pellet was resuspended by vortexing in 100µl of a lysozyme

solution (25mM Tris-HCl buffer pH 8.0, containing 50mM glucose, 10mM EDTA, and 4mg/ml lysozyme which was added just before use). The suspension was allowed to stand at room temperature for 5min before being rapidly mixed (by inverting the tube 2 or 3 times) with 200µl of an alkaline SDS solution (1%(w/v) SDS and 0.2M NaOH) which was less than two weeks old. It was then incubated in ice for 5min. Potassium acetate (150µl of a solution made by mixing 60ml 5M of potassium acetate, 11.5ml of glacial acetic acid, and 28.5ml of double-deionized water, approximately pH 4.8) was added, and the solution vortexed to mix and left on ice for a further 5min. The solution was centrifuged (12,000g for 5min) and the supernatant transferred to a fresh tube. 400µl of a 1:1 mixture of phenol and chloroform were added and mixed by vortexing. The phases were separated by centrifugation (12,000g for 1min), and the upper, aqueous phase transferred to a fresh tube. 800µl of ethanol were added, and the solution mixed by vortexing and incubated at room temperature for 2min. It was then centrifuged (12,000g for 5min), the supernatant being discarded; the pellet was drained, resuspended in 1ml of 70%(v/v) ethanol and recentrifuged (12,000g for 5min). The pellet was dried in a vacuum desiccator for 3min and then redissolved in 50µl TE8 containing pancreatic RNAse (20µg/ml) which had been heated to 90°C for 10min to destroy the DNAse.

3.27 Mini Plasmid Preparations

To test for the presence of plasmid, the mini

plasmid procedure of Kado & Liu (1981) was used.

Bacteria, grown on an agar plate, were scraped off with a sealed end of a capillary tube and transferred to 100µl of lysis buffer (6mg/ml Tris base, 3%(w/v) SDS, 33mM NaOH, approximately pH 12.6) in a 1.5ml microfuge tube which was then incubated at 55°C for 45min. It was cooled to room temperature and 100µl of a 1:1 mixture of phenol and chloroform were added. The solution was vortexed and then centrifuged (12,000g for 5min). The upper, aqueous phase contained DNA, and 30µl of this solution were sufficient to show the presence of pBR322 or related plasmids by agarose gel electrophoresis.

3.28 Quantitation of DNA

The concentration of DNA in a sample was determined by measuring the absorbance of the solution at 260nm. An absorbance of 1 corresponds to approximately 50µg/ml double stranded DNA.

3.29 Agarose Gel Electrophoresis

To separate, identify, and purify DNA fragments, horizontal, submarine gel electrophoresis was employed, using 0.8% agarose slab gels (7.5cm x 9.5cm). Prior to loading, the DNA sample was mixed at a ratio of 2:1 with a solution of 5%(w/v) SDS, 25%(v/v) glycerol, and 0.025%(w/v) bromophenol blue in the electrophoresis buffer. For most purposes, the buffer used for both the gel and aqueous phases was 90mM Tris-borate pH 8.0, 90mM boric acid, 2mM EDTA; electrophoresis was carried out at a potential of 4V/cm until the marker dye had travelled

approximately 6cm. When the DNA was to be recovered from the gel, the buffer used was 40mM Tris-acetate, 2mM EDTA pH 8.0, and electrophoresis was carried out at 1V/cm with recycling of the buffer. Removal of the DNA from the gel by the freeze-thaw method used is facilitated by the use of Tris-acetate rather than Tris-borate as a buffer.

After electrophoresis was discontinued, the electrophoresis buffer was replaced with 1µg/ml ethidium bromide in the same buffer. After 30min, the ethidium bromide solution was removed, the gel washed with double-deionized water; the DNA bands could then be observed under UV light.

3.30 Purification of DNA fragments by the Freeze-thaw Method

DNA fragments in Tris-acetate agarose gels were separated from the gel by a freeze-thaw method.

The area of the gel containing the required fragments was cut out with a fresh razor-blade. It was cut into small pieces with the blade, and these were loaded into a 2ml syringe that had been plugged with 2 Whatman GF/C filters at the bottom of the barrel. The plunger was replaced, and the syringe wrapped in 2 layers of aluminium foil and put into an isopropanol-dry ice bath for 30min. The foil was removed, and the frozen material allowed to thaw slowly over an open 1.5ml microfuge tube. The fluid (about 80% of the total volume) was then carefully pressed out from the syringe. It was washed once with 100µl of phenol (the upper layer being

retained) and 3 times with 100µl of diethyl ether (the bottom layer being retained). The volume of the solution was then reduced to approximately 80µl by mixing with isobutanol which takes up some of the water from the aqueous phase. Isobutanol was added to the aqueous solution, and the 2 phases were mixed thoroughly by vortexing; the phases separated on standing, and the upper, isobutanol phase could be removed, taking with it some dissolved water. By repeating this process several times, the desired volume was be achieved.

At this stage, when the fragment being purified was intended for ligation into pBR322, the appropriately prepared pBR322 (restricted and dephosphorylated) was added to the fragment.

The DNA was precipitated by the addition of 20µl of 1.5M sodium acetate and 600µl of ethanol. After 2h at -70°C, the DNA was collected by centrifugation (12,000g for 5min). The pellet was washed twice with ice-cold ethanol and dried in a vacuum desiccator for 3min. The pellet was dissolved in a small volume of TE8 or in ligation buffer if pBR322 had been added.

3.31 Restriction Endonuclease Digestion

Digests with ECoRl alone were in 100mM Tris-HCl buffer pH 7.5, containing 50mM NaCl & 10mM $MgSO_4$; with HindIII alone, in 50mM Tris-HCl buffer pH 8.0, containing 50mM NaCl & 10mM $MgSO_4$; with both ECoRl and HindIII, the same buffer as for ECoRl alone was used. Alternatively, for digests with both ECoRl and HindIII, the digestion

was carried out sequentially: following a HindIII digestion, the DNA was precipitated by ethanol and sodium acetate (3.32), resuspended, and then digested with ECoRl. Digestion was at 37°C for 1h and was stopped by heating at 70°C for 5min in a boiling-water bath.

3.32 Precipitation of DNA by Ethanol and Sodium Acetate

To 80µl of the DNA sample in a 1.5ml microfuge tube, 20µl of 1.5M sodium acetate and then 600µl of ethanol were added. After 2h at -70°C, the DNA was collected by centrifugation (12,000g for 5min). The pellet was washed twice with ice-cold ethanol and dried for 3min in a vacuum desiccator. It was then dissolved in a small volume of TE8 and stored at -20°C until required.

3.33 Ligation of DNA

T4 DNA ligase was used for the ligation of "sticky-ended" DNA produced by ECoRl and/or HindIII digestion. 1µl of T4 DNA ligase and 3µl of freshly prepared ligase reaction buffer (200mM Tris-HCl buffer pH 7.8, containing 100mM $MgSO_4$, 150mM dithiothreitol, & 12mM ATP) were added to the DNA dissolved in 25µl of 10mM Tris-HCl buffer pH 7.5, 0.1mM EDTA in a 1.5ml microfuge tube. The tube was spun for 5s to mix the solution which was then sealed in glass capillary tubes and incubated at 4°C overnight. The tubes were allowed to warm up to room temperature for about 30min before the DNA was used for transformation.

3.34 Preparation of Multimers of pBR322 to test the ligation procedure

Multimers of pBR322 which can be used as relative molecular mass markers can be prepared from pBR322 that has been digested to completion with a restriction enzyme that cleaves only at one site.

Multimers of pBR322 were prepared from digests with either ECoRl or HindIII in order to test the sucess of the ligation reaction. The digested DNA at a concentration of 500µg/ml was treated as described for ligation. The high concentration of DNA promotes ligation of one molecule of pBR322 to another, producing multimers. After overnight incubation at $4^{o}C$, the products were analyzed by agarose gel electrophoresis. Several bands could be seen with both digestions (Fig. 3.7) showing that the ligation had been successful.

3.35 Transformation of Escherichia coli

Cells were grown to mid log phase in 10ml L-broth. They were collected by centrifugation (5,000g for 5min), resuspended in 5ml of 50mM $CaCl_2$, and incubated at $4^{o}C$ for 30min. The cells were collected by centrifugation as before, and this time , resuspended in 1ml of 50mM $CaCl_2$ and incubated at $4^{o}C$ for at least 1h (the cells were found to be maximally competent after 1h and remained so for at least 24h).

For each transformation, 0.2ml of cells were added to 20ng of DNA in 50µl of buffer. The transformation mixture was incubated at $4^{o}C$ for 30min and then at $40^{o}C$

Figure 3.7: Multimers of pBR322

Mulitmers were prepared as described in 3.34 using (1) an ECoRl digest of pBR322 and (2) a HindIII digest of pBR322.

The bands are, from the bottom of the gel upwards (left to right): linear monomer; closed circular monomer; linear dimer; linear trimer. Other higher relative molecular mass species can also be seen.

for 5min. 1ml of L-broth was added and the mixture incubated at 37°C for 1h. The cells were then collected (5,000g at 4°C for 5min), resuspended in 0.85%(w/v) saline, and plated onto agar.

3.36 Transformation of Bacillus subtilis

B.subtilis was transformed by the method of Wilson & Bott (1968). Cells were grown until late log phase (2.5h after decline in growth rate) in minimal medium containing the Bott & Wilson amino acids (his, arg, val, lys, thr, gly, asp, & met, all 25µg/ml). They were diluted by one third with the same medium, and $MgSO_4$ was added to give a final concentration of 40mM. 0.1ml of DNA (i.e., 6-10µg) was added to 0.9ml of cells for each transformation experiment.

3.37 Nick Translation

Nick translation of the 3.2kb fragment produced by ECoR1 and HindIII digestion of the plasmid, pDB2, was essentially according to the BRL protocol for their nick translation kit. 5µl of solution A2 (0.2mM each of dATP, dGTP, and dTTP, 50mM $MgCl_2$, 100mM 2-mercaptoethanol, and 100µg/ml nuclease-free BSA in 500mM Tris-HCl buffer pH 7.8), 3µl (1µg) of the 3.2kb fragment, 5µl of [α-^{32}P]dCTP (>7,000Ci/mmol), and 32µl of double-deionized water were mixed together in a 1.5ml microfuge tube. 5µl of solution C (0.4u/µl DNA polymerase 1, 40pg/µl DNAse 1, 5mM magnesium acetate, 1mM 2-mercaptoethanol, 0.1mM phenylmethanesulphonyl fluoride, 50%(v/v) glycerol, and 100µg/ml nuclease-free BSA in 50mM

Tris-HCl buffer pH 7.5) was added and the solution mixed gently and spun for 5s at 12,000g in a microfuge. It was then incubated at 15°C for 1h before the addition of 5µl of stop buffer (300mM EDTA pH 8.0).

3.38 Determination of the Amount of Radioactivity Incorporated

The amount of incorporation was tested according to Maniatis et al. (1982) by precipitation with trichoroacetic acid (TCA).

5µl of the sample were spotted onto the centre of a Whatman GF/C glass fibre disc (2.5cm diameter). 5µl of the same sample were also added to a tube containing 100µl of a solution of salmon sperm DNA (500µg/ml in 20mM EDTA). 5ml of ice-cold 10%(v/v) TCA were added and the solution chilled on ice for 15min. The precipitate was collected by filtering the solution through another GF/C glass-fibre disc. The filter was washed 6 times with 5ml of ice-cold 10%(v/v) TCA followed by 5ml of 95% ethanol. Both the filters were dried, put in vials with 5ml of scintillant (0.05%(w/v) PPO + 0.01%(w/v) POPOP) and counted. The first filter measured the total radioactivity in the sample; the second filter measured the radioactivity incorporated into DNA.

Nick translation of the 3.2kb fragment from pDB2 gave an incorporation of 56%.

3.39 Separation of Nick Translated DNA from Unincorporated dNTPs

The radiolabelled DNA was separated from

unincorporated dNTPs by column chromatography as described by Maniatis et al. (1982).

A Sephadex G-50 column was prepared in a 5ml borosilicate glass pipette plugged with sterile glass wool. The column was washed with 5 column volumes of TE8. The translation mixture was applied and 0.5ml/min TE8 run through the column from a reservoir. Fractions of 0.5ml were collected in 1.5ml microfuge tubes. The DNA, excluded from the Sephadex gel, was found in the void volume and detected with a hand-held monitor.

3.40 Southern Blotting

DNA from agarose gels was transferred to nitrocellulose filters by the method of Southern (1975).

After electrophoresis was completed, the gel was placed in a glass baking dish. The DNA was denatured by soaking the gel in several volumes of 1.5M NaCl and 0.5M NaOH for 1h at room temperature with constant shaking. The gel was then neutralised by soaking in several volumes of 1M Tris-HCl pH 8.0, 1.5M NaCl for 1h at room temperature, again with constant shaking. A piece of Whatman 3MM paper was wrapped around a stack of glass plates, and the wrapped support was placed inside a large flat-bottomed baking dish. The support was approximately 1cm larger and wider than the gel. The dish was filled with 10 x SSC (SSC is 150mM NaCl, 150mM Na citrate pH 7.0) almost to the top of the support. The gel was placed with its underside uppermost on the 3MM paper and a piece of nitrocellulose filter (prewet by immersion for 2 to

3min in 2 x SSC), 1 to 2mm larger than the gel in both dimensions, was placed on top. On top of this were placed 2 pieces of Whatman 3MM paper (cut to the size of the gel and wetted in 2 x SSC) and then a stack of paper towels about 5cm high. A glass plate was put on top of the stack, weighed down with a 500g weight.

The transfer of DNA was allowed to proceed for 18h. The nitrocellulose filter was then removed and soaked in 6 x SSC for 5min at room temperature. Excess fluid was allowed to drain from the filter which was then allowed to dry at room temperature on a sheet of 3MM paper. The dried filter was placed between 2 sheets of 3MM paper and baked for 2h at 80^{o}C.

The filter was used in a hybridization experiment as described below.

3.41 Hybridization to Nitrocellulose Filters

Filters were wetted by floating them on the top of 6 x SSC and they were then submerged in the same solution for 5min. They were then incubated at 42^{o}C for 2h in 100ml of prewashing solution (50mM Tris-HCl buffer pH 8.0, containing 1M NaCl, 1mM EDTA, 0.1%(w/v) SDS). The prewashing solution was removed and the filters incubated for 6h at 42^{o}C in 60ml of prehybrization solution (50%(v/v) formamide, 5 x Denhardt's solution, 5 x SSPE, 0.1%(w/v) SDS, and 100µg/ml denatured salmon sperm DNA. Denhardt's solution is 0.2g/l Ficoll, 0.2g/l polyvinylpyrrolidone, 0.2g/l BSA (Pentax Fraction V); SSPE is 250mM NaH_2PO_4 buffer pH 7.0, 150mM NaCl, and 1mM

EDTA).

The radiolabelled probe DNA was denatured by heating for 5min in a boiling water bath and then added to the prehybridization solution covering the filters. Incubation at 42°C was continued for 3h to allow hybridization to occur. The hybridization solution was discarded and the filters washed 3 to 4 times at room temperature, 10min each wash, in 500ml of 2 x SSC and 0.1%(w/v) SDS. They were then washed at 68°C for 1.5h in 300ml of SSC + 0.1%(w/v) SDS. They were dried in air on a sheet of 3MM paper.

3.42 Autoradiography

The dried filters from hybridization were taped to 3MM paper and used to expose Kodak X-Omat S X-ray film until a suitable image was obtained (usually 3-4h at -70°C).

3.43 Colony Hybridization

Bacterial colonies were grown on a nitrocellulose filter that had been placed on the top of an agar plate. (For archaebacteria, which cannot be grown quickly on agar, a small quantity of bacteria was placed directly onto the filter.)

A piece of Whatman 3MM paper was placed on the bottom of each of 4 Pyrex baking dishes. One piece was saturated with 10%(w/v) SDS, excess liquid being removed. The filters were placed colony side up onto the SDS saturated paper and left for 3min. They were then transferred to the second dish with 3MM paper saturated

with denaturing solution (0.5M NaOH, 1.5M NaCl) and left for 5min. The next transfer was to 3MM paper that had been saturated with neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH 8.0) and the filters were left for 5min. After drying for 1h on dry 3MM paper in the last dish, they were sandwiched between two more sheets of 3MM paper and baked at 80^{o}C for 2h.

The radiolabelled probe was hybridized to the filters as described above (3.41).

3.44 Curing of plasmids from Escherichia coli Strains by Ethidium Bromide

100µl of a 10ml overnight culture in L-broth were transferred to 10ml of fresh L-broth + 250µM ethidium bromide and incubated overnight. A 10^{6}x dilution of this culture in 0.85%(w/v) saline was then plated onto L-agar plates. The plates wete incubated overnight and the colonies obtained transferred to both L-agar plates and to L-agar plates + ampicillin. 25% of the colonies obtained from an initially amp^{r} strain (i.e., containing pBR322) were found to be amp^{s}. Mini-plasmid preparations revealed that the amp^{s} clones had been cured of plasmid.

3.45 Dephosphorylation of DNA

Terminal 5'-phosphates were removed from DNA by treatment with calf intestinal alkaline phosphatase (CIP) as desribed by Maniatis (1982).

DNA, digested to completion, was extracted once with phenol/chloroform and precipitated with ethanol. It was dissolved in a minimum volume of 10mM Tris-HCl pH

8.0, and 5µl of CIP buffer(0.5M Tris-HCl buffer pH 9.0, containing 10mM $MgCl_2$, 1mM $ZnCl_2$, and 10mM spermidine), and 0.01U of CIP per pmol of DNA was added. The total volume was made up to 48µl with double-deionized water. The solution was incubated at 37°C for 30min and then 40µl of water, 10µl of 10 x STE (STE is 10mM Tris-HCl buffer pH 8.0, containing 100mM NaCl and 1mM EDTA), and 5µl of 10%(w/v) SDS were added. It was heated to 68°C for 15min and then extracted twice with phenol/choroform and twice with choroform. It was then passed through a spun column of Sephadex G-50 as described below. The DNA obtained was precipitated with ethanol. After resuspension in a small volume of TE8, it was used for ligation.

3.46 Spun-column Procedure

A column (0.9ml bed volume) of Sephadex G-50, equilibrated in STE, was prepared in a 1ml disposable syringe plugged with sterile glass wool. The Sephadex was packed by centrifuging the column at 1600g for 4min inside a tube in a bench centrifuge. The DNA sample was applied in a volume of 0.1ml and the column was spun again, collecting the effluent in a decapped 1.5ml microfuge tube.

3.47 Mutagenesis with Ethyl Methanesulphonate

Cells from 20ml of an early log-phase culture were suspended in 10ml of 100mM Na/K PO_4 buffer, pH7.0. 100µl of ethyl methanesulphonate were added and the suspension incubated at 37°C for 20min. The cells were collected by centrifugation and resuspended in 20ml of

L-broth. They were incubated at 37°C overnight to allow recovery of the culture.

3.48 Mutagenesis with Ultraviolet Light

Cells from 5ml of an early log-phase culture in L-broth (about 2×10^8 cells/ml) were suspended in 2.5ml 0.1M $MgSO_4$ and transferred to a 3.5cm diameter sterile petri dish. The lid of the dish was removed and the cells exposed to 5×10^{-5} J/mm^2 of UV radiation (254nm) over a period of 1min from a previously calibrated source. A cell survival rate of about 0.5% was obtained using these conditions.

3.49 Selection for Glutamate Auxotrophs

A sample of a mutated culture was inoculated into minimal medium with succinate as carbon/energy source and with 2mM glutamate. The culture was grown to stationary phase and the cells used to inoculate fresh succinate-glutamate medium to a density of 10^6 cells/ml. This culture was allowed to grow to early log phase. The cells were collected by centrifugation, washed once in succinate minimal medium without glutamate, and finally resuspended in the same medium. Methicillin (20μg/ml) was then added and the culture incubated for 1h. The cells were again collected by centrifugation and this time washed and then resuspended in succinate-glutamate medium. The whole process was then repeated. The resulting culture was serially diluted and plated onto L-agar. The colonies obtained were picked onto succinate and succinate-glutamate minimal agar to detect glutamate auxotrophs.

4. THE METHODS OF ASSAY OF CITRATE SYNTHASE

4.1 Introduction

Investigation of any physico-chemical system requires the existence of at least one sound method of making measurements of that system. All measurements of a system yield imperfect results, being biased by the method of measurement. This bias can be reduced by making measurements in as many different ways as possible. By such an approach, information that may be obscured or lost in one method becomes available in another.

In enzymology, the availability of a simple and reproducible assay provides a powerful tool for the detection, purification, and kinetic studies of an enzyme, and a great deal of information may be obtained. But, for a fuller understanding of an enzyme system, in a variety of different conditions (temperature, pH, solvent, ±attenuators), more than one method of assay is required.

There are two general types of method: continuous, where the reaction is monitored throughout, and discontinuous, where the reaction is allowed to proceed unmonitored, but samples (or the whole reaction mixture) are tested at appropriate, recorded times. Continuous assays are preferable, since they allow a better estimate of initial rate and any peculiarity in the progress curve of the reaction can be more easily detected. Discontinuous assays do sometimes have an

advantage in that the presence of possible inhibitors or inactivators in the assay mixture is avoided during the course of the reaction.

All methods rely on detectable physical or chemical differences between substrates and products; the reaction can be monitored either by following the disappearance of substrates or the appearance of products. Following the appearance of products is preferred, since a greater relative change in the measured quantity is obtained.

As well as being an enzyme with a central role in metabolism (1.2.1), one of the main reasons why CS has been so well studied is that several methods of assay are available, most importantly including a simple, continuous spectrophotometric assay using DTNB (3.4.1). This chapter contains a discussion of the various assays that have been used for CS; the relative advantages and disadvantages are compared and the limits of each assay noted. Also, a new discontinuous assay has been developed using 2,4-dinitrophenylhydrazine (DNPH) to measure OA utilization. This assay can be used in the presence of free thiols, such as CoA.

4.2 Assays Involving the Measurement of Coenzyme A

4.2.1 Continuous Assay Using DTNB (Srere, 1969; 3.4.1)

This assay is by far the best of those available, and it is responsible for the advanced state of knowledge of CS. It is a simple and rapid method,

involving the addition of only one compound (DTNB) other than the substrates and the enzyme preparation. It is a sensitive method: as little as 10μU CS can be measured routinely; 1mU/ml gives an absorbance change of 0.0136 units per min at 412nm. The method can also be adapted for use as a rapid and sensitive assay for OA or AcCoA. Nevertheless, as stated above, no method is perfect, and there are three main disadvantages to this assay. Firstly, DTNB is known to inactivate some CSs; fortunately, with many CSs, the degree of inactivation is not significant in the time scale of the assay. The second disadvantage, closely related to the first, is that DTNB will react with any free SH groups present, for example, those on a protein (this is the reason for the inactivation of CSs by DTNB), and so in crude extracts, there is likely to be a large background reaction; more importantly, the reaction cannot be studied in the presence of thiols (e.g., CoA, making product inhibition studies impossible). Finally, the pH range of the assay is limited to 7.4 to 9.0, because the yellow-colour of the thio nitrobenzoate anion is only expressed in this pH range. Fortunately, this pH range covers the optimum of CSs, at least as far as the forward reaction is concerned.

4.2.2 The Discontinuous Assay with DTNB

The first of the problems mentioned in 4.2.1 can be avoided by using a discontinuous assay, adding DTNB after the reaction has proceeded for a measured

time. The discontinuous assay was found to give the same values for the activity of preparations of E.coli, Bacillus megaterium, and pig heart CSs as the continuous assay.

4.2.3 The Polarographic Assay (Weitzman, 1966b)

CoA, but not its S-acyl derivatives such as AcCoA, produces an anodic polarographic wave at a dropping mercury electrode; this is the basis for a polarographic assay of CS. The procedure involves the continuous monitoring of the appearance of the anodic wave, which can be performed automatically by a recording polarograph. No chromogenic reagent is introduced, and so there is no possibility of inactivation of the enzyme by such a compound. It works well with crude extracts; any slight turbidity introduced does not interfere with the measurements, unlike a spectrophotometric assay.
It can measure down to about 1mU of CS, i.e., 100 x less sensitive than the DTNB method; 1mU/ml gives a change of 1.63nA. Though it can be used in the presence of some thiols, it is limited to low concentrations of CoA or other compounds that will give a signal at the same potential.

4.2.4 Oxidation of Coenzyme A by Dichlorophenol-indophenol (Wieland et al., 1964b)

This assay has been almost universally ignored, possibly since the only account of it is in German. It is based on the reduction of dichlorophenol-indophenol (DCPIP) by free CoA, reduction of DCPIP being followed by

the change in absorbance at 578nm (e_{578} 12,700 $l.mol^{-1}.cm^{-1}$). It suffers from the same disadvantages as the DTNB assay, in that a potentially inhibitory chromogen is added to the reaction, and any free thiols initially present will cause a reduction of the DCPIP. DCPIP is also a less specific reagent than DTNB, taking part in reactions other than with free SH groups. The absence of compensatory advantages could also be a reason for its disuse.

4.3 Assays Involving Measurement of Citrate

4.3.1 Radiochemical Method Using ^{14}C Acetyl-coenzyme A (Wieland et al., 1964a & b)

Whilst potentially very powerful methods of assay, radiochemical assays require expensive radiolabelled substrates, the assays take longer to perform and involve a greater number of manipulations than spectrophotometric methods, thus increasing the influence of random errors. They are of necessity discontinuous methods: the reaction must be stopped and then the radiolabelled products and substrates separated before the radioactivity is measured. In the case of the assay for CS using radiolabelled AcCoA, radiolabelled citrate is separated from AcCoA by its precipitation as a silver salt.

4.3.2 By Bromination of Citrate

This method is based on the observation that citric acid yields pentabromoacetone on bromination (Cahours, 1847). Addition of thiourea then yields a

coloured complex that is yellow on alkalination (pH 11) and can be measured spectrophotometrically (λmax = 460nm). This method has been used to detect citrate (Natelson et al., 1948), and has been used with the $AcPO_4$ assay (4.5). However, there is no report of its use in an uncoupled assay, though in theory, it could be used in this way. Because bromination of the reaction mixture is required, it is unlikely to ever find routine use.

4.4 Utilization of Acetyl-coenzyme A

(Ochoa, 1957; Srere & Kosicki, 1961)

The thioester bond of AcCoA absorbs at 232nm, and so there is a decrease in absorbance at this wavelength as the citrate synthase reaction proceeds. Along with the polarographic method (4.2.3), it is the most straightforward method in that only the reaction components are present. The high absorbance of proteins and many other compounds make it unsuitable for use with crude extracts. Because of the nature of the assay, the lower the AcCoA concentration the better, the upper limit being about 0.4mM. This is clearly undesirable in a study of CS where saturating concentrations of AcCoA are required.

4.5 Utilization of Acetyl Phosphate (Ochoa, 1955)

This old fashioned assay was employed before the method of preparation of AcCoA was described. It involves the coupling of the CS rection to the transacetylase reaction:

$$AcPO_4 + CoA \rightleftharpoons AcCoA + PO_4 \text{ (transacetylase)}$$

$$AcCoA + OA + H_2O \rightleftharpoons citrate + CoA + H^+ \quad (CS)$$

The transacetylase and the CoA were provided by a preparation of E.coli that was substantially free of CS and aconitase. The decrease in $AcPO_4$ was measured by the hydroxamate method of Lipmann & Tuttle (1945), or alternatively, the formation of citrate was measured by the bromination method described in 4.3.2. The AcCoA is formed in situ, and so its steady state concentration will be low and cannot be varied. Because of the low concentration of AcCoA, the reaction rates measured are lower than with other methods. This assay offers no advantages over the modern methods and is included only for completeness.

4.6 Assays Using Malate Dehydrogenase

The following methods depend upon the fact that OA is a product of the MDH reaction as well as a substrate for the CS reaction. The two reactions are of course consecutive reactions of the citric acid cycle.

$$L\text{-malate} + NAD^+ \rightleftharpoons OA + NADH + H^+ \quad (MDH)$$

$$AcCoA + OA + H_2O \rightleftharpoons citrate + CoA + H^+ \quad (CS)$$

4.6.1 Continuous Assay With Malate Dehydrogenase (Ochoa, 1955)

Like the $AcPO_4$ assay (4.5), this involves the generation of one of the substrates (this time OA) in situ. Therefore in this assay, the steady state concentration of OA is low and cannot be varied, and so the reaction rates are lower than for other methods. The formation of NADH is followed at 340nm or with greatly

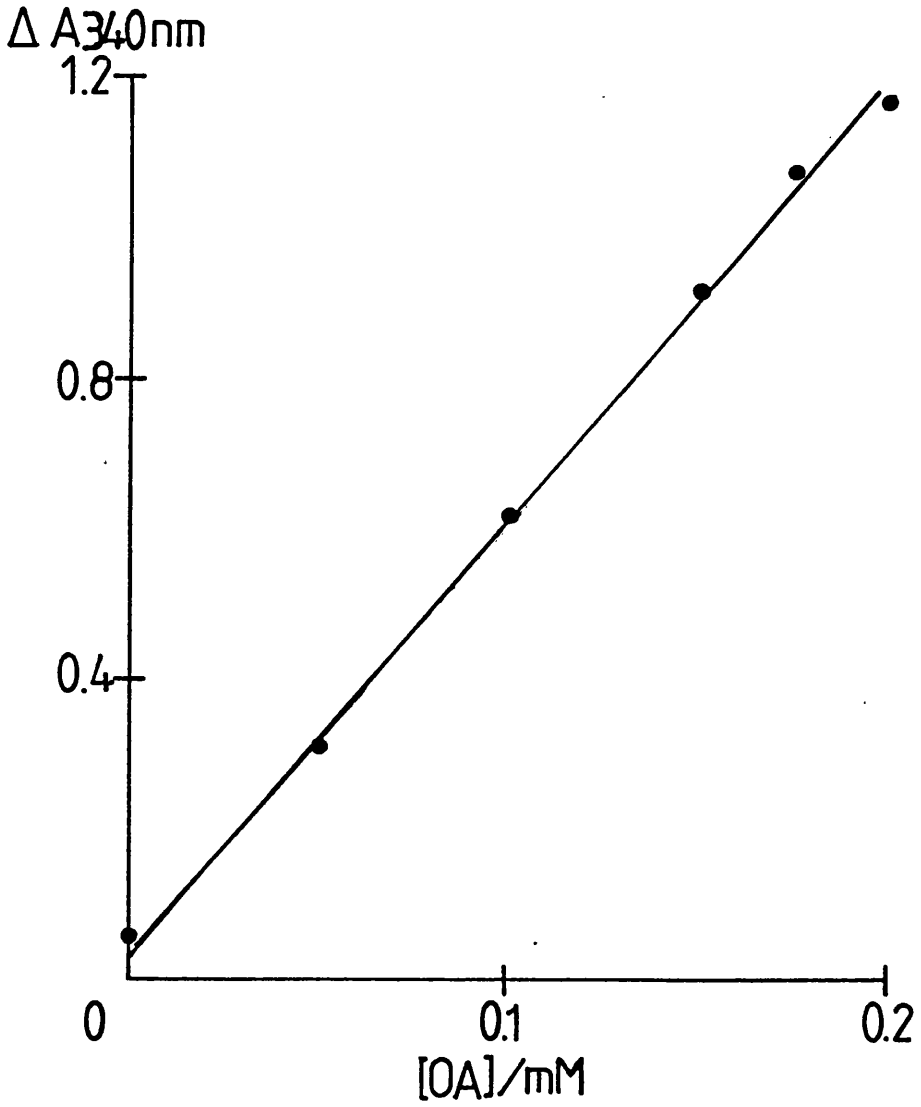
enhanced sensitivity by fluorimetry (Shepherd & Garland, 1966). This assay was used to measure E.coli CS. It was found that the reaction profiles were markedly curved (approaching steady state), and consequently it was difficult to determine an initial or even a steady state rate from them. A further problem with this assay is that NADH is an inhibitor of some CSs (e.g., that of E.coli), and its presence in the assay is likely to affect the result.

4.6.2 Discontinuous Assay With Malate Dehydrogenase

In the same way that the DTNB assay can be adapted to work in a discontinuous mode, the MDH coupled assay can also be used discontinuously. Although there are no reports on the use of this assay, it was found that in this case, the resulting assay is better than the associated continuous one. The linearity of measurement of OA by MDH is shown in Fig. 4.1.

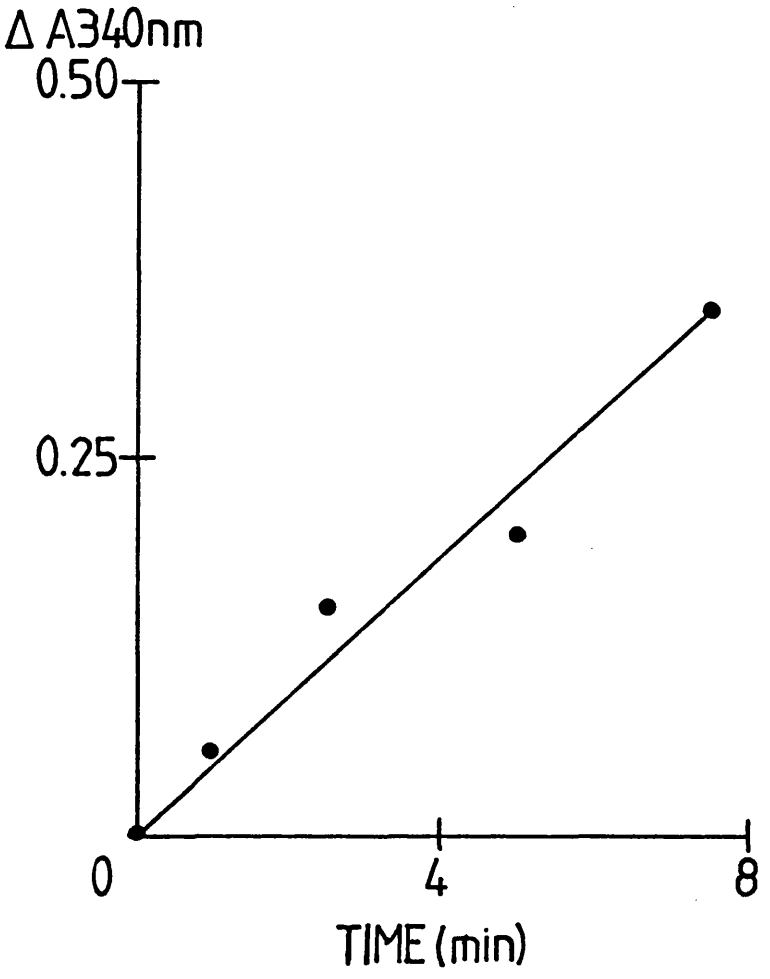
An acid-alkali treatment is used to stop the CS reaction: addition of 20µl of 1M HCl to 1ml of reaction mixture, in order to destroy all the enzyme activity, followed by addition of 20µl of 1M NaOH in order to return the solution to pH 8.0. Heat cannot be used, since OA is destroyed, whereas it is stable to the acid-alkali treatment. With E.coli CS, the reaction is approximately linear for at least 7.5min (Fig. 4.2). A time of 3min was chosen to study the linearity of the assay with respect to the amount of enzyme added (Fig. 4.3). It was found to be approximately linear up to an absorbance change per

Figure 4.1: Standard Curve for the Measurement of Oxaloacetate by Malate Dehydrogenase



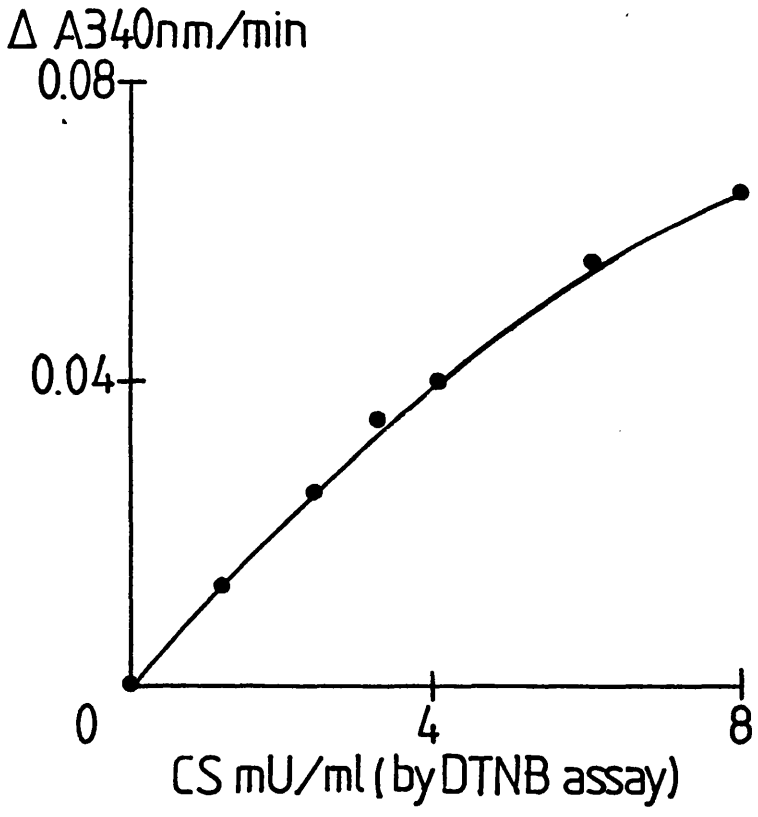
The reaction was in ET8 buffer containing 0.3mM NADH, 0.6U/ml MDH, and OA as indicated. It was started by the addition of the MDH. The small absorbance of MDH at 340nm was accounted for.

Figure 4.2: Time Course of the Reaction as Measured by Following the Disappearance of Oxaloacetate with Malate Dehydrogenase



The CS reaction was in ET8 buffer containing 0.2mM OA, 0.15mM AcCoA, and was started by the addition of 6mU/ml of E.coli CS. The reaction was stopped by the addition of 20µl of 1M HCl at the times indicated. 20µl of 1M NaOH were then added, followed by NADH to a final concentration of 0.3mM. 0.6U of MDH was added, and the change in absorbance at 340nm was measured after the reaction had gone to completion.

Figure 4.3: Linearity of Discontinuous Malate Dehydrogenase-linked Assay for Citrate Synthase with Respect to Enzyme Concentration



The protocol was the same as for Fig. 4.2, except that a time of 3min was used througout, and the amount of E.coli CS added was varied.

min at 340nm of about 0.04 (i.e., 6.4mU/ml CS). In comparison, the DTNB assay was linear with respect to enzyme up to at least an absorbance change per min at 412nm of 0.165 (i.e., 12mU/ml CS), and gave activities approximately 1.75 x those from the discontinuous assay with MDH.

The discontinuous assay with MDH avoids any possible effects of NADH on CSs. The assay has three major disadvantages:-

1. It requires the MDH reaction to go to completion, and so accurate measurements of start and end points are necessary.
2. Addition of acid and alkali must be carefully controlled, in order to prevent errors caused by differences in volume and/or pH from one assay to the next
3. The disappearance of small amounts of OA from a relatively high concentration of that compound are difficult to measure; the assay is therefore relatively insensitive.

4.6.3 Assay of Citrate Synthase in Reverse by Coupling to Malate Dehydrogenase

The equilibrium position of the CS reaction lies in favour of the formation of citrate (Keq = 1.2 x 10^{-4}M in PO_4 buffer at 22°C - Stern et al., 1952), whereas the equilibrium position of the MDH reaction lies in favour of malate formation (Keq = 6.4 x 10^{-13}M in Tris buffer at 25°C - Yoshida, 1965). Therefore, it should be

possible to drive the CS reaction in reverse by coupling it to the MDH reaction. Low pH and high citrate concentration will also favour the reversal of the CS reaction. Kosicki & Srere (1961) used 100mM imadazole-acetate, pH 6.1, as a buffer, and 50mM citrate. With pig heart CS, they found that the assay gave values of Vmax about 4% of those of the 232nm assay of the forward reaction. The decrease in absorbance was linear for at least 3min when the rate was less than 0.04 absorbance units per min, and there was a linear dependence on enzyme concentration up to the same rate. Again, the presence of NADH could be a disadvantage of this assay. With crude extracts, the presence of NADH oxidase would be a problem, though it can sometimes be eliminated by cyanide.

4.7 Assay of Citrate Synthase by measurement of Oxaloacetate Disappearance with 2,4-dinitrophenylhydrazine

4.7.1 Introduction

This method was developed in order to be able to measure CS accurately in the presence of thiols, specifically for the purposes of the experiments described in chapter 5 that involved cleaving the thioester bonds of palCoA and palthio (5.2.6). The products included free thiols, and these interfere with reagents such as DTNB. Clearly, the polarographic assay cannot be used, since this measures free CoA, and the assay at 232nm measures the absorbance of thioester bonds

and is therefore of no use. The MDH linked assays could in theory be used, but neither the continuous nor the discontinuous assays are very reliable; they both have various defects as discussed above (4.6.1 & 4.6.2).

The DNPH assay described here has two disadvantages: it is discontinuous, and it involves the measurement of a substrate and so is relatively insensitive. These disadvantages are sufficient to prevent its routine use in place of the DTNB assays, but it was ideal for the use for which it was designed.

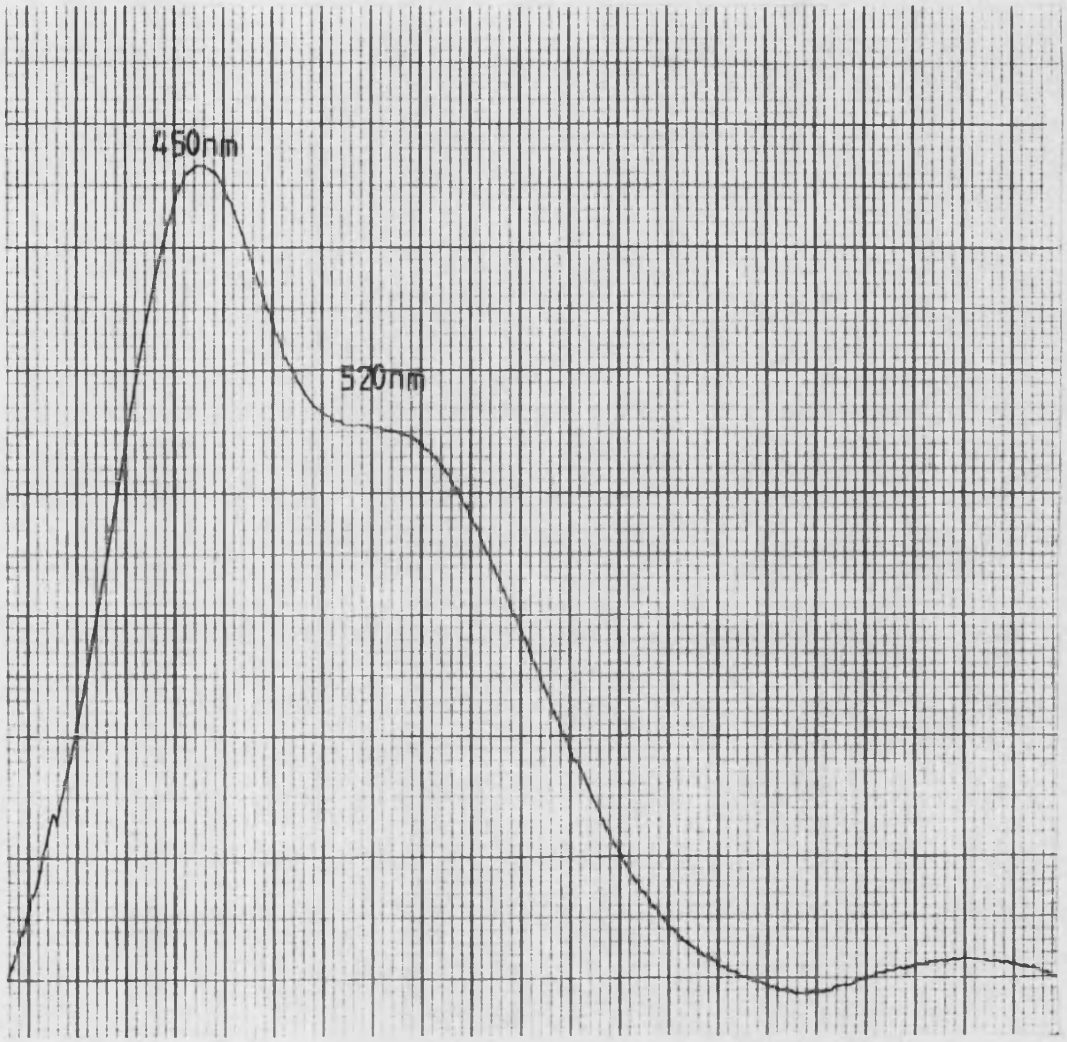
4.7.2 Formation of a 2,4-dinitrophenylhydrazine derivative of Oxaloacetate

DNPH in acid solution reacts with the ketone group of OA to give a yellow coloured 2,4-dinitrophenylhydrazone derivative. It is difficult to measure this derivative spectrophotometrically, since DNPH is itself yellow coloured, but if the solution is made alkaline, then a brick red coloured compound is produced, and this compound can be measured spectrophotometrically. In the spectrum of this compound, shown in Fig. 4.4, there is a peak at 450nm. This wavelength was used for all the following studies.

4.7.3 Time Course of the Reaction

120µl of 10mM OA were added to 3ml of 0.5mM DNPH in 1M HCl. At recorded times, 0.5ml samples were withdrawn and added to 0.5ml of 2M NaOH. The absorbance of the resulting solution was measured immediately and also after 30min and 150min. The results are shown in

Figure 4.4: Spectrophotometric Scan of the 2,4-dinitrophenylhydrazone of Oxaloacetate in Alkaline Solution



The scan was from 370nm to 800nm (left to right). 1mM OA gives a derivative with an absorbance of 12.5 at 450nm.

Fig. 4.5. The reaction was essentially complete after 10min, and the compound formed was relatively stable. In the experiments that follow, a 10min incubation period was used, and the absorbances were recorded 5min after the addition of NaOH.

4.7.4 Test of Possible Interferences

DNPH reacts with aldehyde or ketone groups, and therefore it reacts with OA. It would not be expected to react with AcCoA, CoA, citrate, or with palCoA, palthio, and palmitate. These substances were tested to ensure that they did not react, and as expected, none of these substances produced a coloured derivative. Nevertheless, this assay would not be suitable if high concentrations of aldehydes or ketones were required in the assay mixture. Fortunately, this is not usually the case with CS.

4.7.5 Estimation of Oxaloacetate

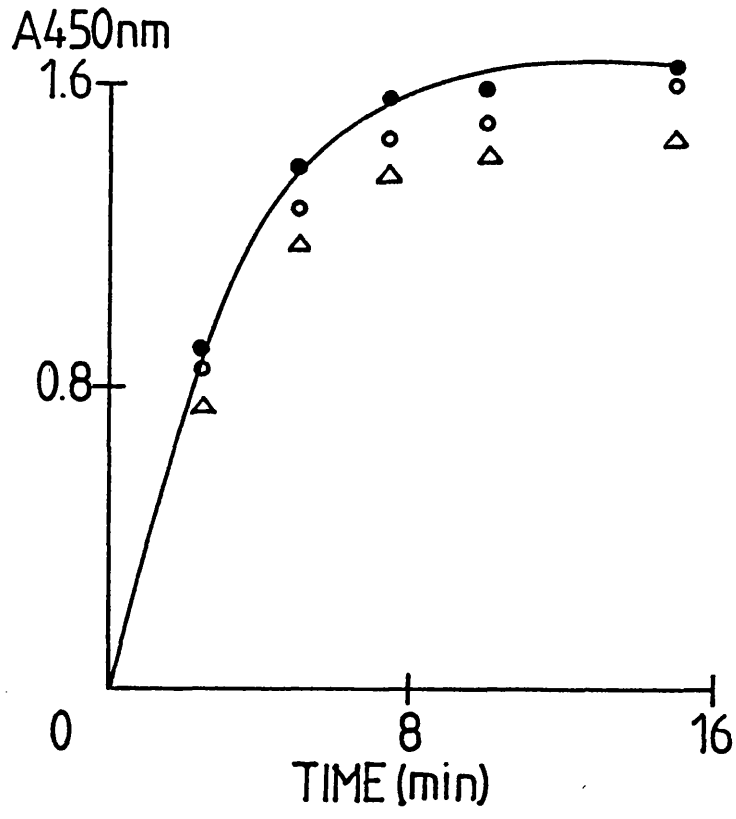
Variation of final OA concentration (i.e., the concentration after the addition of the alkali) up to 100µM produced a linear response in the absorbance at 450nm (Fig. 4.6). Assuming a 100% conversion of OA into the coloured derivative, this gives an absorption coefficient of 12,500 $l.mol^{-1}.cm^{-1}$ for the derivative.

4.7.6 Assay of Citrate Synthase

4.7.6.1 Time Course of the Citrate Synthase Reaction

40µl of 10mM OA and 40µl of 7.5mM AcCoA were added to 1.82ml of ET8 buffer. The reaction was started by the addition of 100µl of Bacillus megaterium CS (70mU

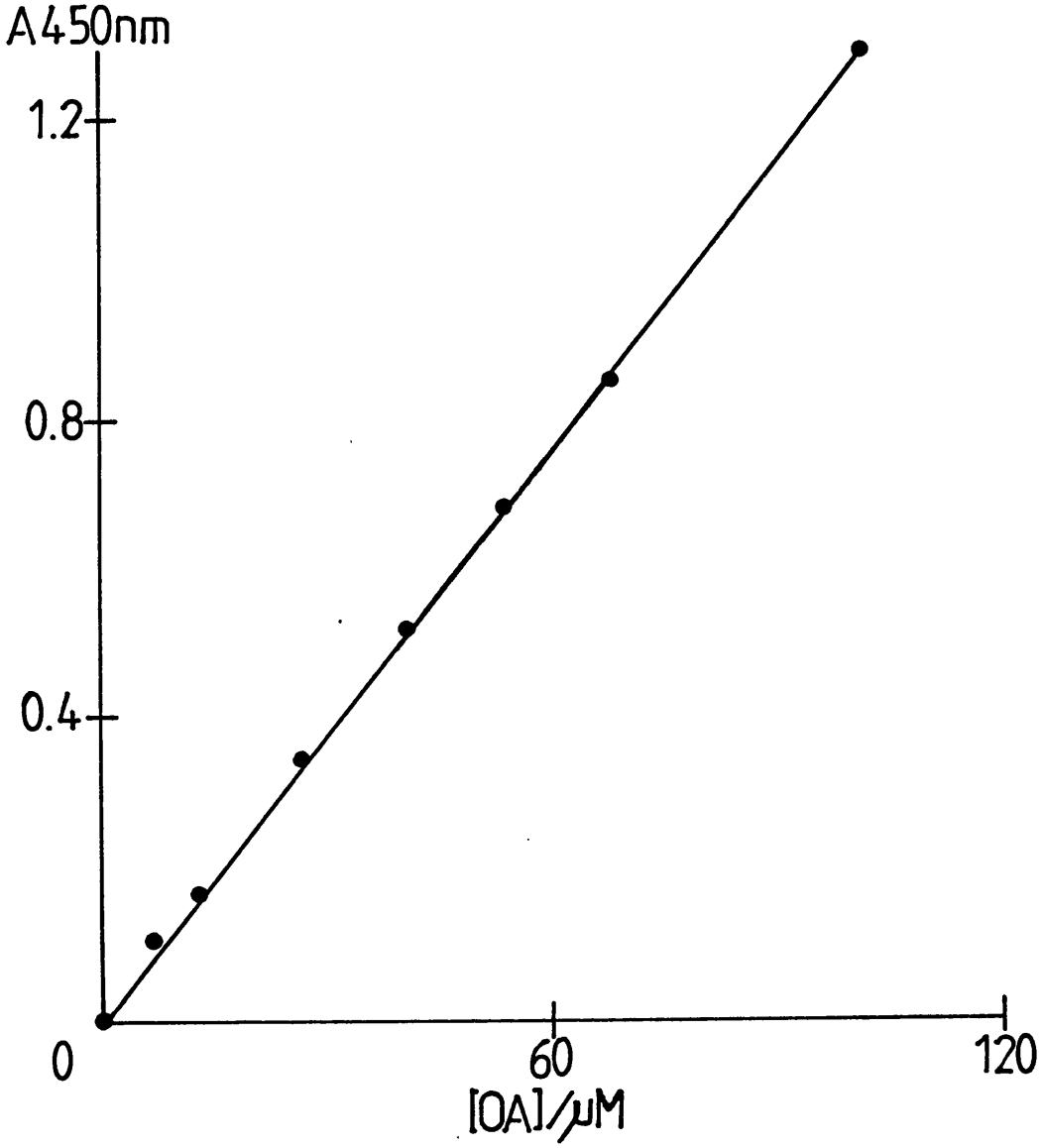
Figure 4.5: Time Course of the Reaction of 2,4-Dinitrophenylhydrazine with Oxaloacetate



The reaction was carried out as described in 4.7.3.

- ● readings taken immediately
- o readings taken after 30min
- △ readings taken after 150min

Figure 4.6: Measurement of Oxaloacetate by Reaction with 2,4-Dinitrophenylhydrazine



OA was incubated in 0.5ml of DNPH (0.5mM in 1M HCl). After a 10min incubation, 0.5ml of 2M NaOH was added, and the aborbance at 450nm was recorded after a further 5min incubation.

by DTNB assay) and the reaction mixture was incubated at room temperature. 50µl samples were withdrawn every minute for 5min, and added to 0.45ml of 0.5mM DNPH in 1M HCl, this solution was left for 10min before the addition of 0.5ml of 2M NaOH. After a further 5min, the absorbance at 450nm of the alkaline solution was recorded.

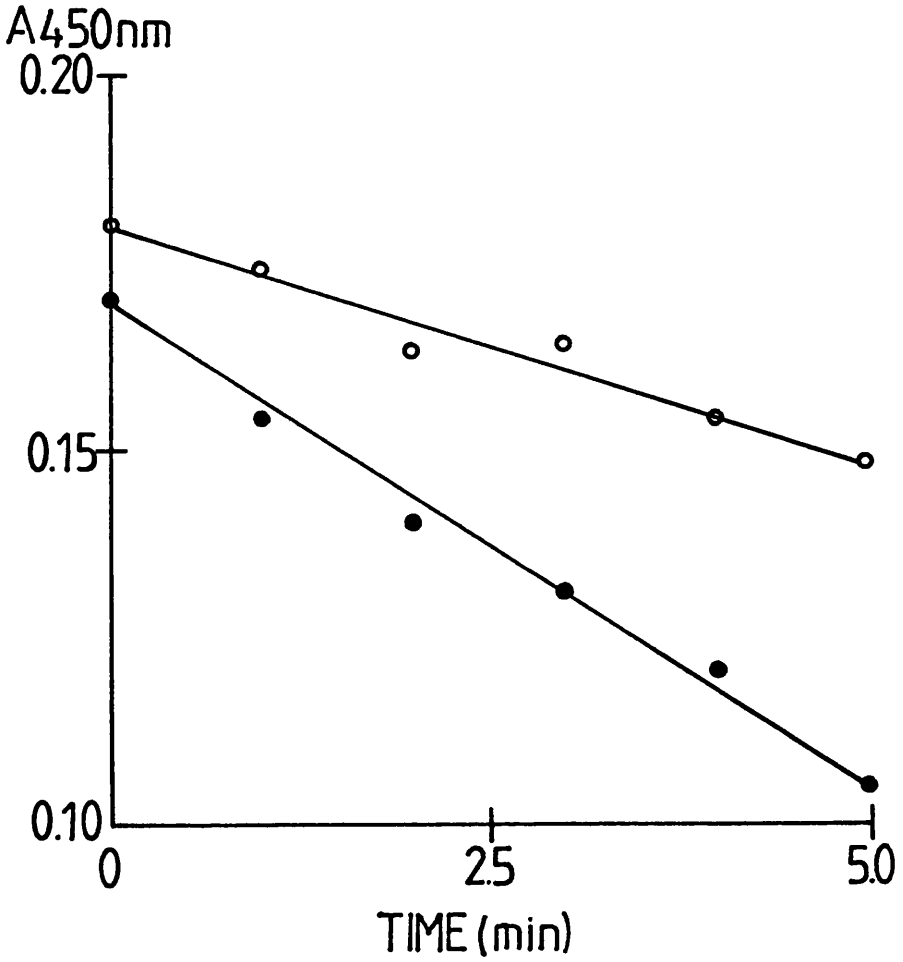
The experiment was repeated using 200µl (35mU) of CS, and the results of both experiments are shown in Fig. 4.7. The reactions were approximately linear over the periods recorded, and gave a value for the activity of CS approximately 59% of those obtained by the DTNB assay. The doubling of enzyme added caused a doubling in the rate measured. The linearity with respect to enzyme was further tested as described in the next section.

4.7.6.2 Linearity With Respect to Enzyme Concentration

The following procedure was used as an assay for CS in order to test the linearity with respect to enzyme concentration.

A reaction mixture containing 0.2mM OA, 0.15mM AcCoA, and enzyme (Bacillus megaterium CS) in ET8 buffer was incubated for 3min at 25°C. 100µl of the mixture were then added to 0.4ml of DNPH and incubated at 25°C for 10min. 0.5ml of 2M NaOH was added, and the absorbance of the solution at 450nm was recorded after a further 5min. From Fig. 4.8, it can be seen that the change in absorbance is linear with repect to enzyme concentration up to about 56mU of enzyme per ml of the original reaction mixture.

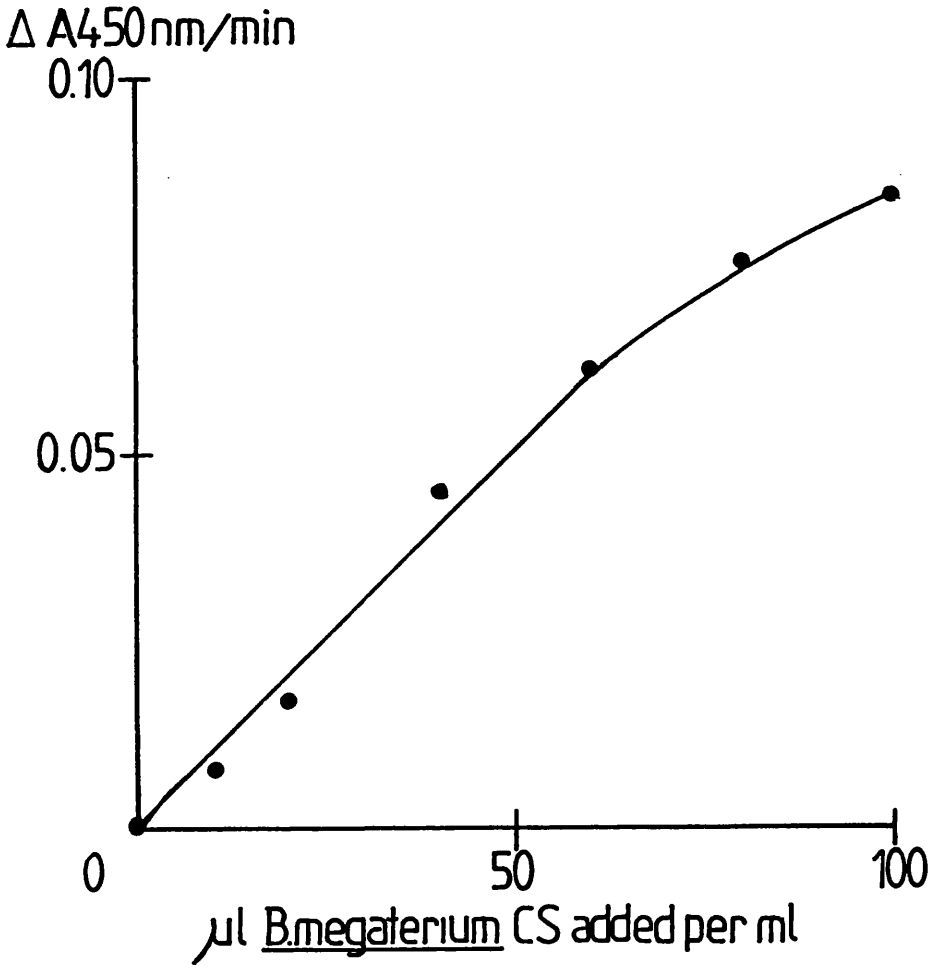
Figure 4.7: Time Course of the Citrate Synthase reaction as Measured by Oxaloacetate disappearance Using 2,4-Dinitrophenylhydrazine



The reaction was carried out as described in 4.7.6.1.

- o 100µl B.megaterium CS
- • 200µl B.megaterium CS

Figure 4.8: Linearity of the 2,4-Dinitrophenylhydrazine Assay With Respect to Enzyme Concentration



The reaction was carried out as described in 4.7.6.2.

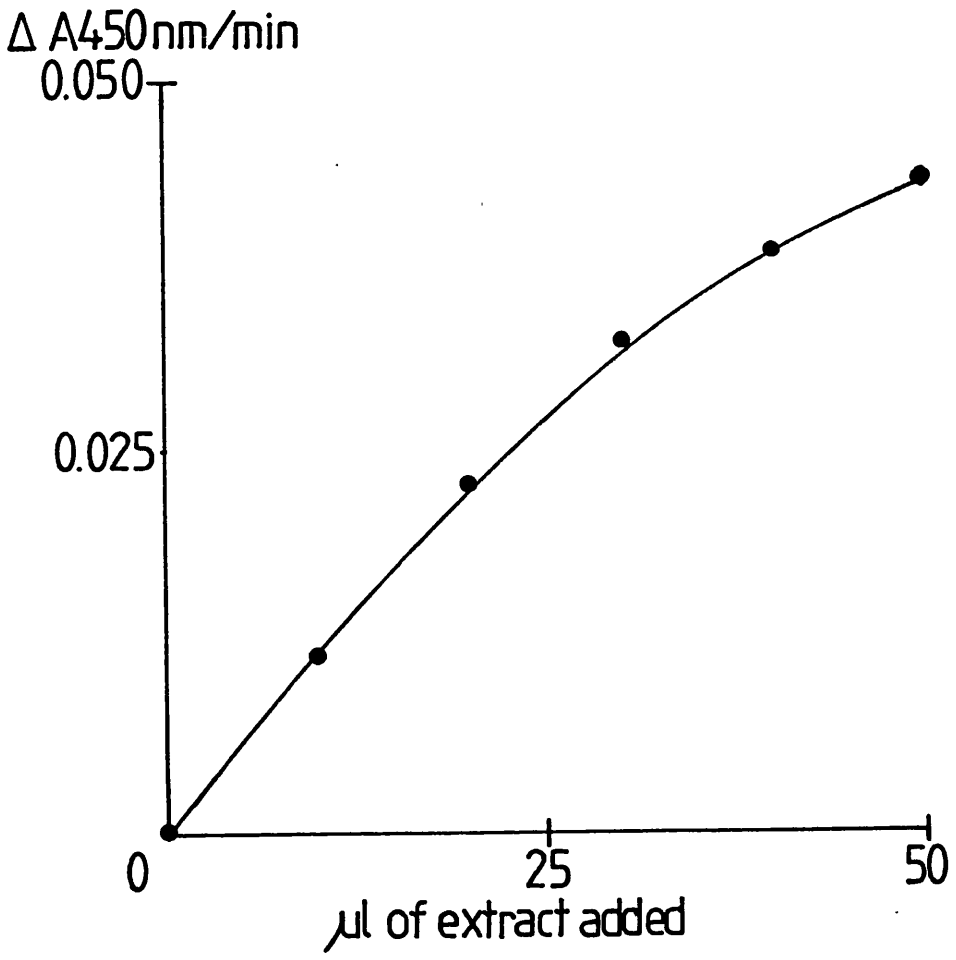
4.7.6.3 Use of the Assay

The assay was used for the experiment described in 5.2.6. It can be used for the assay of CS in crude extracts (Fig. 4.9). It is a valuable addition to the assays of CS, allowing assays in the presence of free thiols, and thus enabling studies on the effects of thiols on CS.

4.8 Concluding Comments

The assays discussed above represent only a small number of the possible ways in which the CS reaction could be monitored, but they include all the major methods that have been used so far. All of the assays have defects, but some offer no compensatory advantages over any of the other assays (e.g., the $AcPO_4$ assay and the continuous MDH linked assay). The method of choice in most circumstances is the continuous DTNB assay, but several other assays find use in circumstances where the DTNB assay cannot be used (e.g., the DNPH assay in the presence of thiols, and the polarographic assay at low pH). With the exception of the experiment described in 5.2.6, the continuous DTNB assay was used throughout this work.

Figure 4.9: Use of 2,4-Dinitrophenylhydrazine to Assay Citrate Synthase in a Crude Extract of E.coli, K12



The reaction was carried out as described in 4.7.6.3.

5. THE EFFECTS OF PALMITOYL-COENZYME A AND RELATED COMPOUNDS ON CITRATE SYNTHASE

5.1 Introduction

5.1.1 Inhibition of Fatty Acid Synthesis by Fatty Acyl-coenzyme A

In the animal tissues that have been investigated, the overall process of fatty acid synthesis and a number of individual reactions in the pathway, e.g. acetyl-CoA carboxylase (EC 6.4.1.2), are inhibited by fatty acyl-CoAs such as palCoA (Porter & Long, 1958; Tubbs & Garland, 1963; Bortz & Lynen, 1963a). Moreover, changes in diet or hormone levels cause inverse changes in palCoA and fatty acid synthesis, consistent with a role for palCoA as a feedback inhibitor (Garland & Tubbs, 1963; Bortz & Lynen,1963b).

5.1.2 Mammalian Citrate Synthase is Inhibited by Fatty Acyl-coenzyme A

As discussed briefly in 1.5, mammalian CS is also inhibited by fatty acyl-CoA. The inhibition occurs at concentrations of palCoA (5×10^{-6}M) that could be found *in vivo*. The kinetics of the inhibition are complex: it is non-competitive with respect to AcCoA, sigmoidal with respect to palCoA, time dependent (Wieland & Weiss, 1963) and is apparently competitive with respect to OA (Wieland *et al.*, 1964a). These and other aspects of the inhibition are discussed in detail below.

5.1.3 Palmitoyl-coenzyme A inhibition of Citrate Synthase as a Possible Control Mechanism in Fatty Acid Synthesis and Ketogenesis

In animals, citrate is intimately connected with fatty acid synthesis: it is the provider (via ATP citrate lyase, EC 4.3.1.8) of the cytosolic AcCoA (the precursor for fatty acid synthesis), and it allosterically activates AcCoA carboxylase, the first enzyme of the pathway. It has therefore been suggested that palCoA acts as a feedback inhibitor of CS in order to regulate fatty acid synthesis (Tubbs, 1963); high palCoA levels would result in a reduction of citrate formation, and this in turn would cause a decrease in cytosolic AcCoA and a decrease in the activity of AcCoA carboxylase; thus the rate of fatty acid synthesis would be reduced.

It has also been suggested that high palCoA, by switching off CS and fatty acid synthesis, would divert AcCoA for acetoacetate formation (Tubbs, 1963; Wieland & Weiss, 1963). The concentration of palCoA in the liver is expected to be high in conditions leading to ketogenesis. In this context, inhibition of CS in heart muscle by high palCoA would be detrimental. However, Wieland & Weiss (1963) suggest that this is explained by a 10-fold lower level of palCoA in the heart as compared to liver, and so inhibitory concentrations of palCoA might not be present at the site of CS in the heart.

5.1.4 Objections to Palmitoyl-coenzyme A as a Physiological Regulator of Citrate Synthase

Srere (1965) and others (e.g., Taketa & Pogell, 1966) have suggested that the inhibition of CS by palCoA is not of physiological significance and that it is due merely to non-specific detergent effects: palCoA is known to be a good detergent (Zahler et al., 1968). Several pieces of evidence supported this suggestion:-

1. Srere (1965) pointed out that all the available physiological evidence at the time showed that increases in palCoA were associated with increases in citrate, directly opposite to what would be expected if palCoA regulated citrate formation.
2. When a molar excess of palCoA was added to CS, 16 molecules of palCoA were bound per enzyme molecule. Srere (1965) proposed that the inhibition could be caused by the physical blocking of the active site by the CoA parts of the bound palCoA (several molecules of palmitate also bind but without causing any inhibition). However, the data do not preclude the possibility that one of the palCoA molecules binds specifically, whilst the others bind non-specifically. It is worth noting that the aggregation number for palCoA in micelles is about 40 (Powell et al., 1981), i.e., greater than the number of molecules bound per enzyme molecule.
3. Inhibition occurs at concentrations approximately equal to the critical micelle concentration (cmc) of palCoA. This was true when the cmc was believed to be

3-4μM, but more recent studies indicate that the cmc is an order of magnitude higher than this (30-60μM)(Powell et al., 1981); inhibition therefore occurs at concentrations an order of magnitude below the cmc and so cannot be due to micellar effects.

4. The interaction is dependent not on the palCoA concentration, but on the molar ratio of palCoA to CS. Results described here shed light on this observation and an explanation is proposed (5.2.2).

5. Several enzymes such as glutamate dehydrogenase, which are unrelated to fat metabolism, are inhibited by palCoA (Taketa & Pogell, 1966). The properties of the inhibition are almost identical from enzyme to enzyme. This is a very powerful argument against the rationale concerning control of fatty acid synthesis and ketogenesis. It suggests that the interaction(s) between palCoA and CS is not specific.

5.1.5 Work with Analogues of Fatty Acyl-coenzyme A Suggests that there is a Specific Interaction with Citrate Synthase

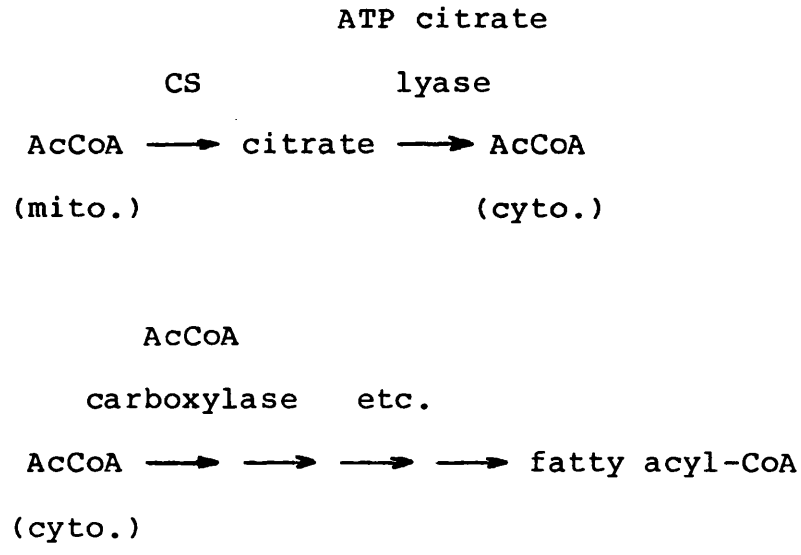
Oleoyl-(1,N^6-etheno)-CoA, an analogue of oleoyl-CoA differing in the adenine moiety of the CoA, was found to be a slightly better detergent than oleoyl-CoA, but it was a worse inhibitor of CS by an order of magnitude (Hsu & Powell,1975). Competitive binding studies with the spin-labelled fatty acyl-CoA analogues, 6-doxylstearoyl-CoA and 16-doxylstearoyl-CoA, show that AcCoA and ATP do not compete for the binding

site, but OA and NADPH compete very effectively (Caggiano & Powell, 1979). The fatty acyl chain of the bound fatty acyl-CoA was found to have a degree of motional freedom.

5.1.6 Physiological Rationale for Palmitoyl-coenzyme A Inhibition of Citrate Synthase in Bacteria

The physiological rationale in terms of control of ketogenesis cannot be applied to bacteria, since they do not synthesize ketone bodies.

The rationale in terms of fatty acid synthesis is based on the operation of the following pathway:



The breakdown of citrate to give AcCoA is catalyzed by ATP citrate lyase (EC 4.1.3.8). This enzyme does not exist in most bacteria; in those that it does, it is involved in a reductive citric acid cycle (Shiba et al., 1985). There are therefore two alternative routes from AcCoA to fatty acyl-CoA via AcCoA carboxylase:-

1. AcCoA ⟶ fatty acyl-CoA

i.e., one pool of AcCoA available to by both CS and AcCoA carboxylase.

2. AcCoA(pool 1) ⟶ citrate $\xrightarrow{\text{CITRATE LYASE}}$ acetate

acetate ⟶ AcCoA(pool 2) ⟶ fatty acyl-CoA

i.e., two pools of AcCoA, one available to CS and one to AcCoA carboxylase.

Control of fatty acid synthesis by inhibition of CS would only make sense if the second pathway were the one in operation. Citrate is an allosteric activator of mammalian AcCoA carboxylase. Again, in bacteria, activation by citrate would only make sense if the second pathway were in operation. E.coli AcCoA carboxylase is unaffected by citrate; this is circumstantial evidence for the first pathway. It should be borne in mind that, although in animals fatty acids are principally used as a reserve source of energy, in bacteria they are primarily incorporated into phospholipids. Different mechanisms of regulation can therefore be expected. Also, alternative pathways have been proposed for the formation of malonate in bacteria, for example, via aspartate and malonyl semi-aldehyde (Al-Ssum & White, 1977).

5.1.7 Aims of the present Work

The significance of the effects of fatty acyl-CoA on CS is the most controversial aspect of current work on CS. The comparative study undertaken here was an attempt to clarify the situation.

Physiological rationales in terms of control of

ketogenesis have no significance in bacterial systems, and so if the observed effects of palCoA on mammalian CSs are also observed in bacterial systems then this hypothesis becomes less plausible. As discussed in 5.1.6, the consequences for the rationale in terms of fatty acid synthesis can only be decided when the pathway from AcCoA to fatty acids in bacteria is elucidated.

In addition, it was hoped that information concerning structure-function relationships in CSs would be obtained from such a study.

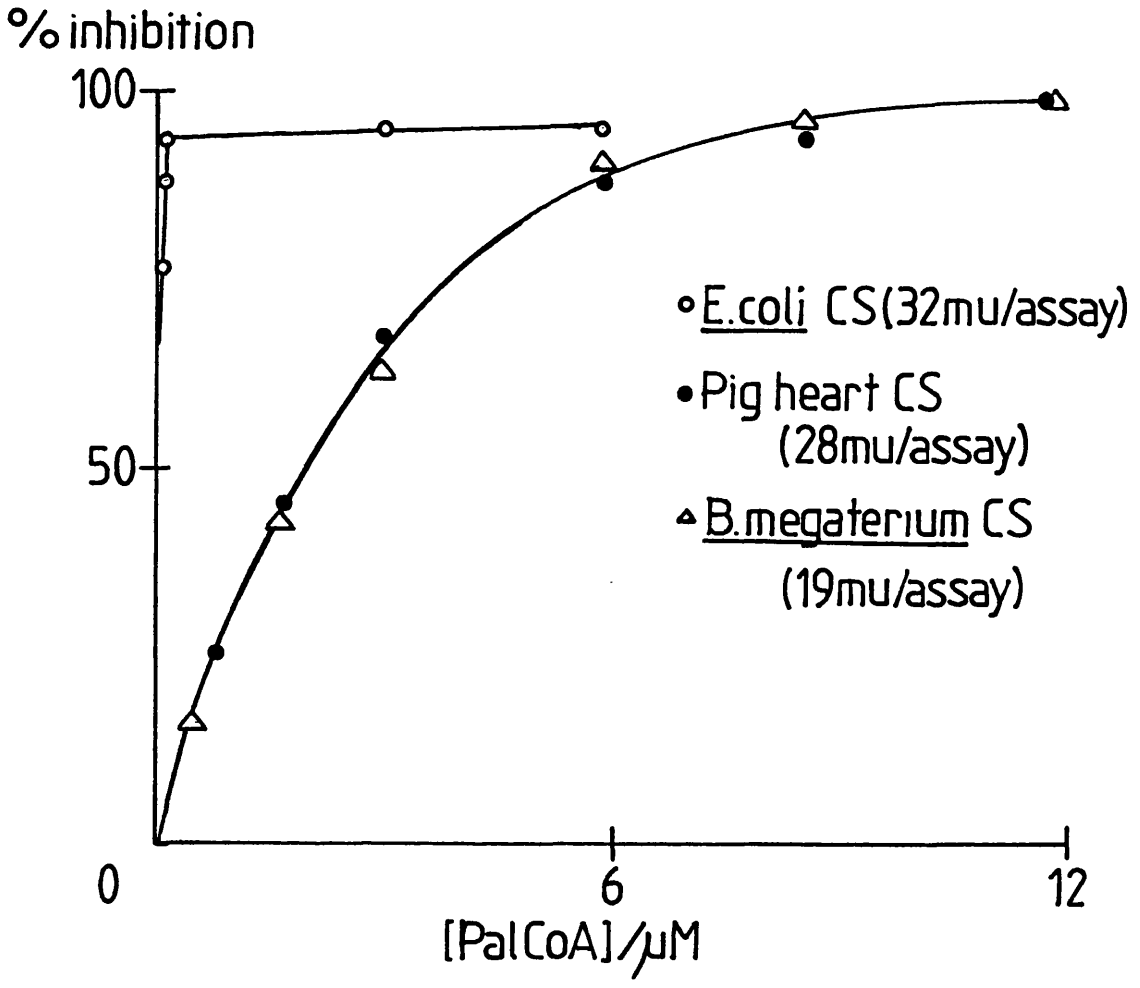
5.2 Results and Discussion

All the reactions described here were carried out at 25°C.

5.2.1 Inhibition of Citrate Synthases by Palmitoyl-coenzyme A

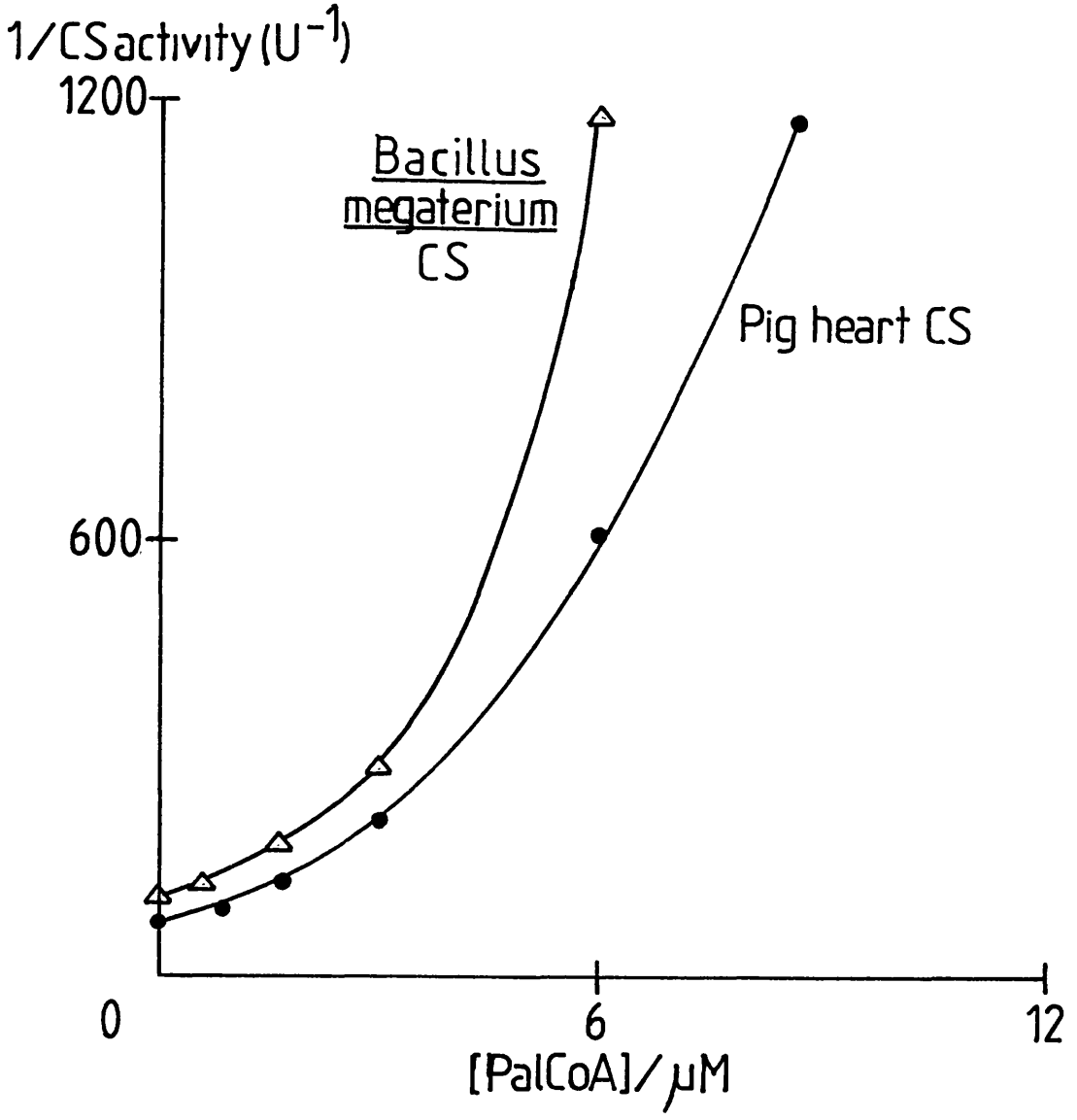
In assays of CS without preincubation of the reaction mixture, palCoA was found to inhibit E.coli, Bacillus megaterium, and pig heart CSs (Fig. 5.1). The Bacillus megaterium enzyme behaved identically to the pig heart enzyme, 50% inhibition being obtained at approximately 2μM palCoA. E.coli CS was even more sensitive - 50% inhibition at approximately 0.03μM palCoA. Dixon plots of the inhibition curved upwards in all three cases (Figs. 5.2 & 5.3); such a curve would be obtained by co-operative binding of an inhibitor. The presence of 100mM KCl in the assay buffer caused a slight desensitization of E.coli to palCoA inhibition (50%

Figure 5.1: Palmitoyl-coenzyme A Inhibition of the Citrate Synthases of E.coli, Bacillus megaterium, and Pig Heart



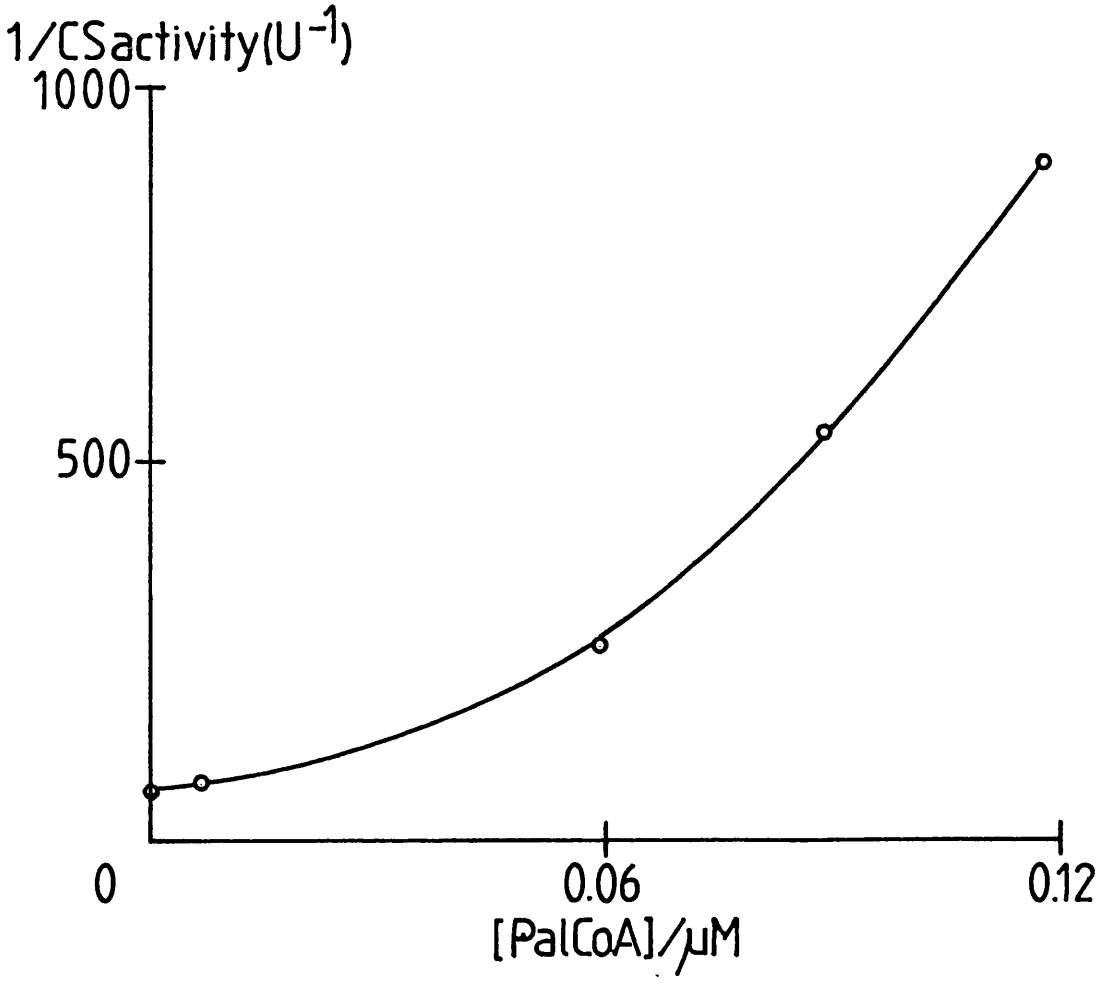
The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction. All assays were in ET8 buffer.

Figure 5.2: Dixon plot of the Inhibition of Bacillus megaterium and Pig Heart Citrate Synthases by Palmitoyl-coenzyme A



The data were plotted according to the method of Dixon (1953). The assays were carried out as described in 3.4.1. There was no preincubation of the enzyme and inhibitor. Enzyme was added to start the reaction.

Figure 5.3: Dixon plot of the inhibition of E.coli Citrate Synthase by Palmitoyl-coenzyme A



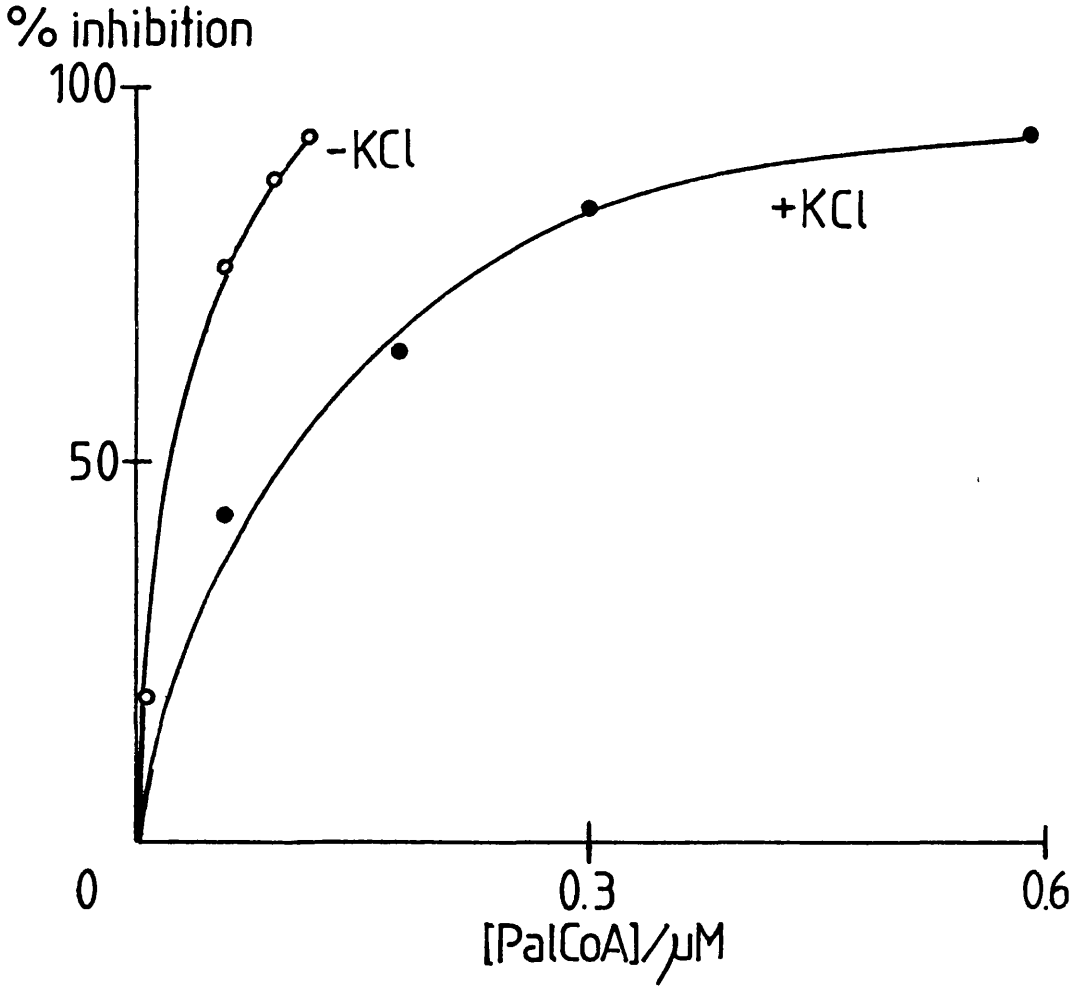
The data were plotted according to the method of Dixon (1953). The assays were carried out as described in 3.4.1, using ET8 buffer. There was no preincubation of the enzyme and inhibitor. Enzyme was added to start the reaction.

inhibition at approximately 0.1μM)(Fig. 5.4) in agreement with earlier studies (Srere, 1968). However, in contrast to Srere's results, variation of enzyme concentration over a limited range (3-14 mU/ml) did not significantly affect the degree of inhibition of any of the three enzymes (Figs. 5.5-5.7). Also at odds with earlier work is the lack of sigmoidicity observed in plots of palCoA v percentage inhibition (Figs. 5.1, 5.2, & 5.5-5.7). Only in the case of E.coli CS with the lowest amount of enzyme (3.5 mU/ml) was there any noticeable degree of sigmoidicity. The data of Hsu & Powell (1975) on the oleoyl-CoA inhibition of pig heart CS do not give sigmoid plots. The earlier sigmoidal data are those of Wieland & Weiss (1963) and Srere (1965), but the latter data may not be reliable as the amount of enzyme quoted could not have been detected by the DTNB assay used.

5.2.2 Further Studies on the Effect of Palmitoyl--coenzyme A on Bacillus megaterium Citrate Synthase

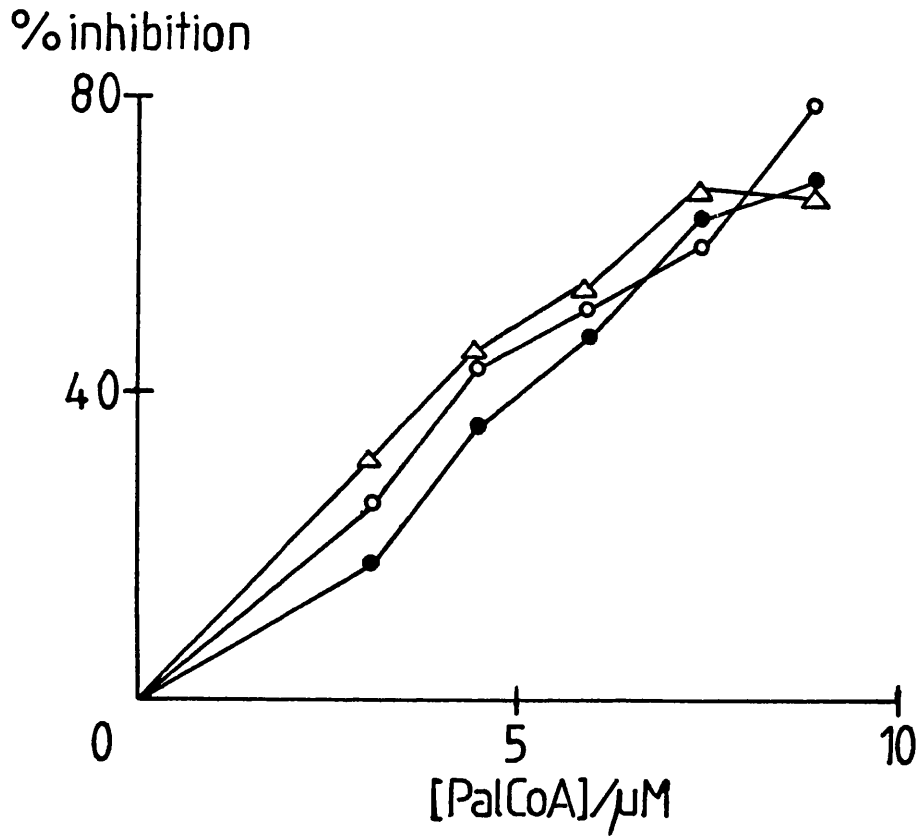
Bacillus megaterium CS was found to behave towards palCoA in exactly the same way as pig heart CS. The inhibition is both time dependent and irreversible (i.e., it is really inactivation); in this case, the relative amount of CS to palCoA did appear to have some effect (Figs. 5.8 & 5.9). In previous studies on the pig heart enzyme, CS had been measured after preincubation of the enzyme with palCoA. If the molar ratio is important in the time dependent inactivation, but not for the inhibition per se, then the discrepancies pointed out in

Figure 5.4: Inhibition of E.coli Citrate Synthase by Palmitoyl-coenzyme A in the Presence and Absence of KCl



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

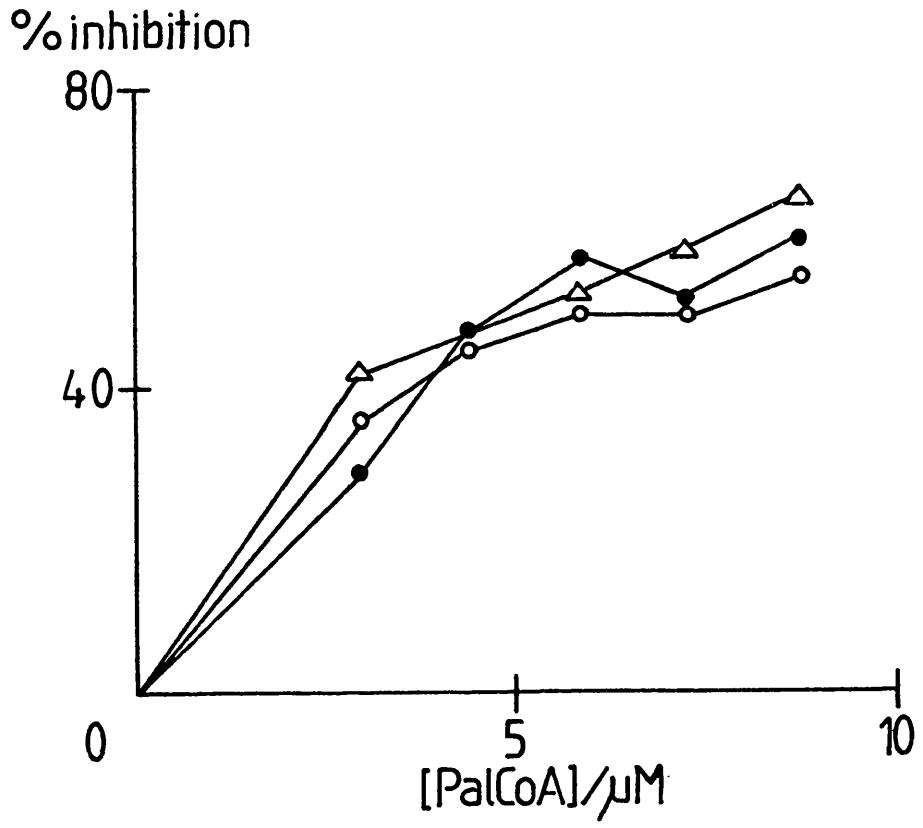
Figure 5.5: Inhibition of Bacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A: Variation of Enzyme and Inhibitor Concentrations



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

- ● 14mU CS
- ○ 7mU CS
- △ 4mU CS

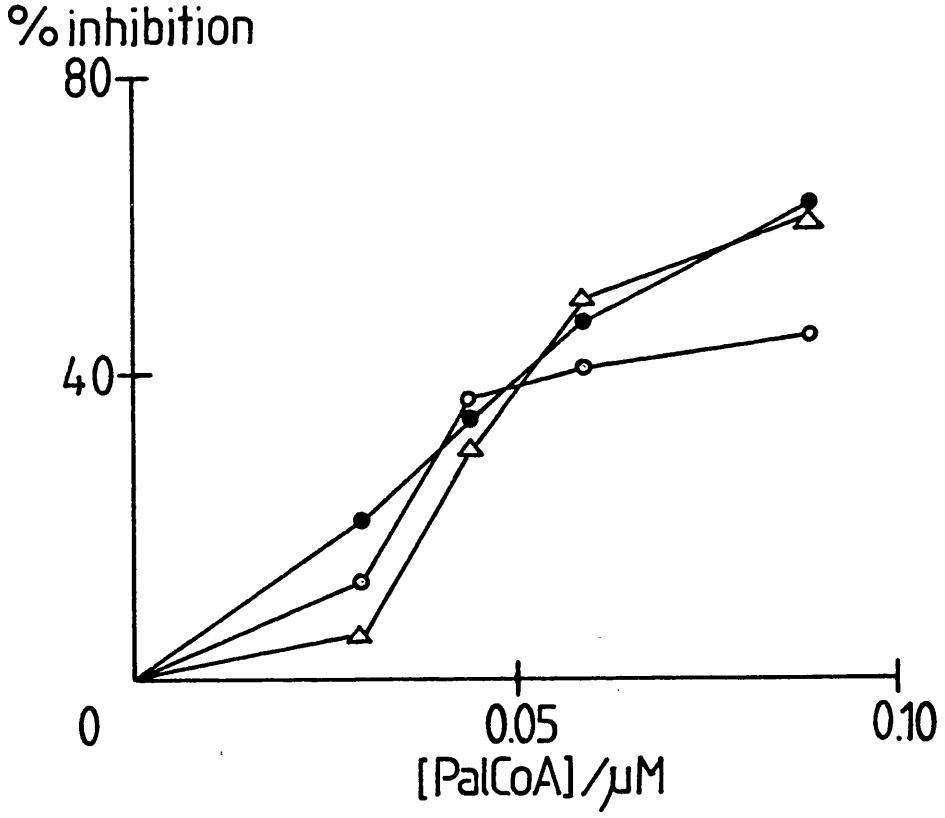
Figure 5.6: Inhibition of Pig Heart Citrate Synthase by Palmitoyl-coenzyme A: Variation of Enzyme and Inhibitor Concentrations



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

- ● 13mU CS
- o 7mU CS
- △ 4mU CS

Figure 5.7: Inhibition of E.coli Citrate Synthase by Palmitoyl-coenzyme A: Variation of Enzyme and Inhibitor Concentrations

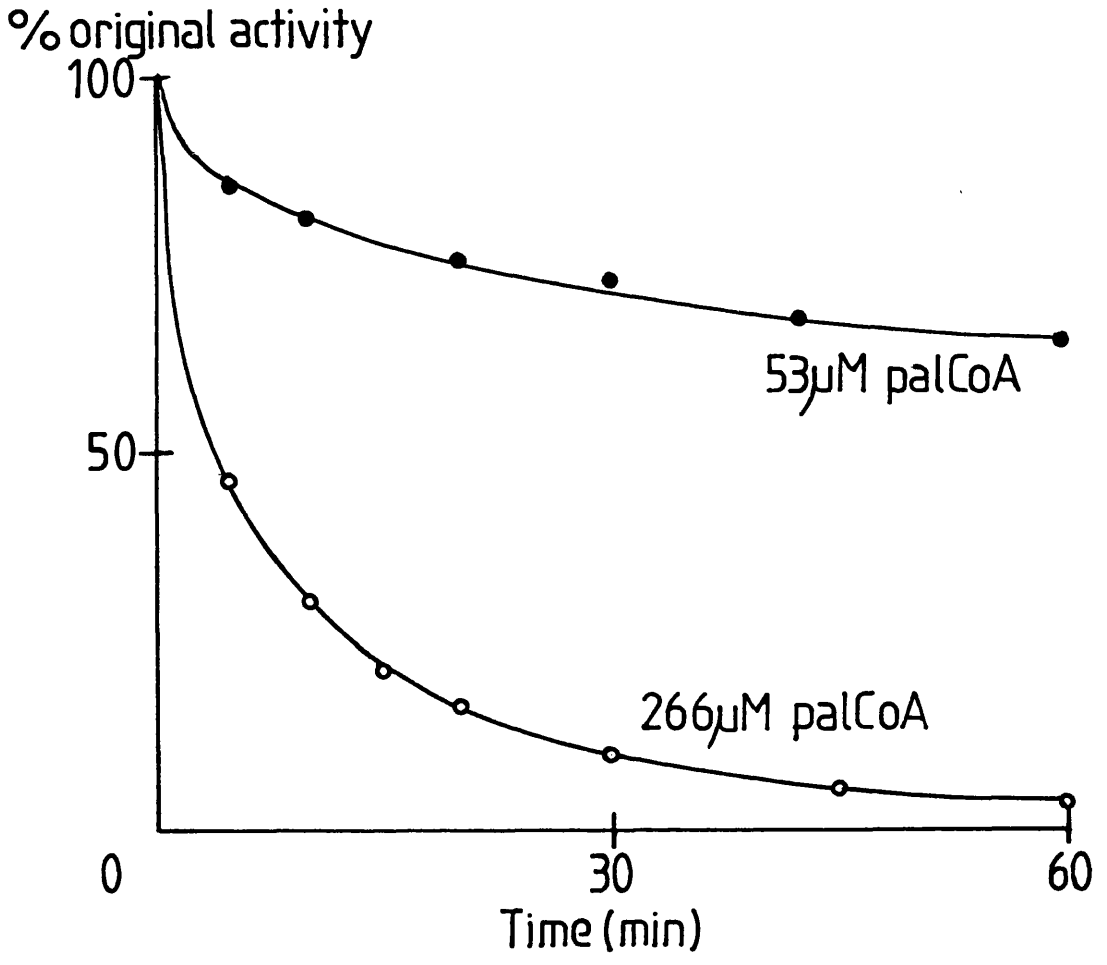


The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

● 12mU CS
o 7mU CS
△ 4mU CS

Figure 5.8: Inactivation of Bacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A with Time

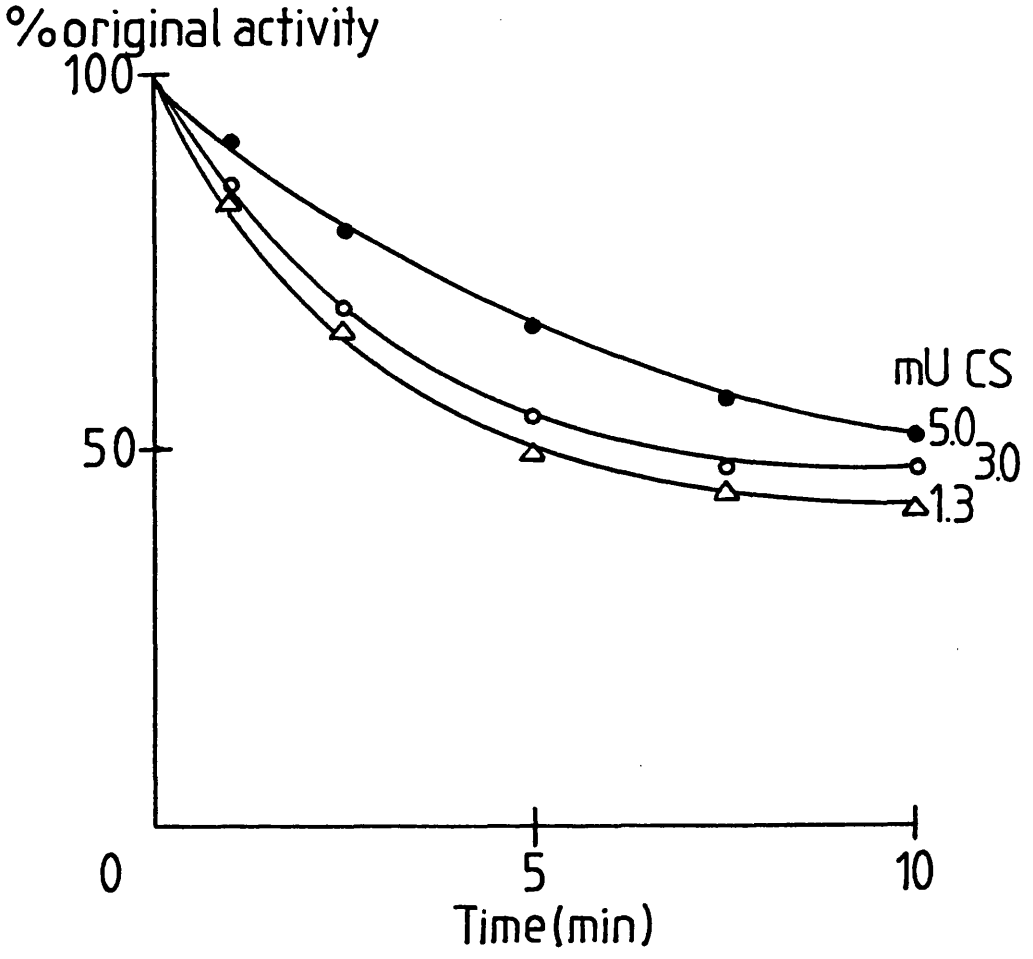
(1) Variation of Palmitoyl-coenzyme A Concentration



200µl of B.megaterium CS (647mU) were added to 200µl of palCoA. 10µl samples were withdrawn, at the times indicated, and assayed for CS as described in 3.4.1. The concentrations of palCoA shown in the figure refer to the concentration after the addition of the CS.

Figure 5.9: Inactivation of Bacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A with Time

(2) Variation of Enzyme Concentration



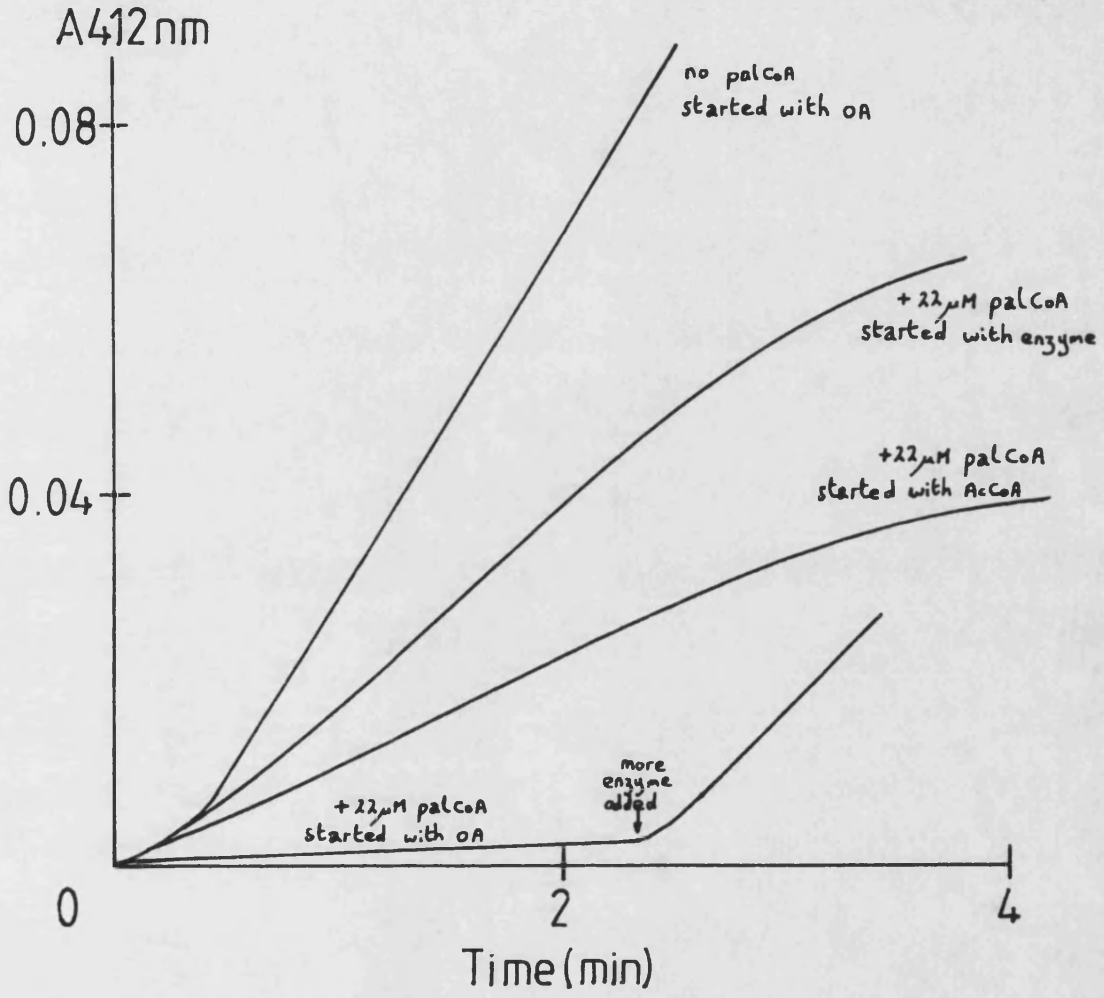
The assays were carried out as described in 3.4.1. Enzyme and inhibitor were added to the buffer and incubated for the time indicated before the addition of AcCoA and OA. The final concentration of palCoA used throughout was 4.5μM.

5.2.1 can be explained. A possible explanation of such effects takes into account the multiple binding of palCoA to CS that has been observed. Assuming that inhibition is determined by the inhibitor concentration, then with relatively high concentrations of enzyme with respect to palCoA, the palCoA concentration could be reduced with time because of relatively slow binding to the high concentration of hydrophobic enzyme binding sites. With relatively low concentrations of enzyme with respect to palCoA, the change in palCoA concentration would be smaller. The result would be that after preincubation less inhibitor would be present in free solution, and the higher the enzyme:inhibitor ratio the greater the change. High enzyme concentrations would therefore encounter less inhibitor than low enzyme concentrations; hence the apparent effect of the molar ratio on the inhibition after preincubation.

Other effects of preincubation are manifested in the different rates obtained when the components are added in different orders (Fig. 5.10). Almost 100% inhibition was seen when the reaction was started with OA (i.e., enzyme incubated with palCoA and AcCoA); this exemplifies the large protective effect of OA against palCoA inhibition, it is not however 100% effective. AcCoA would seem to provide a degree of protection, but much less than OA. These results are almost identical to those of Srere (1965) with pig heart CS.

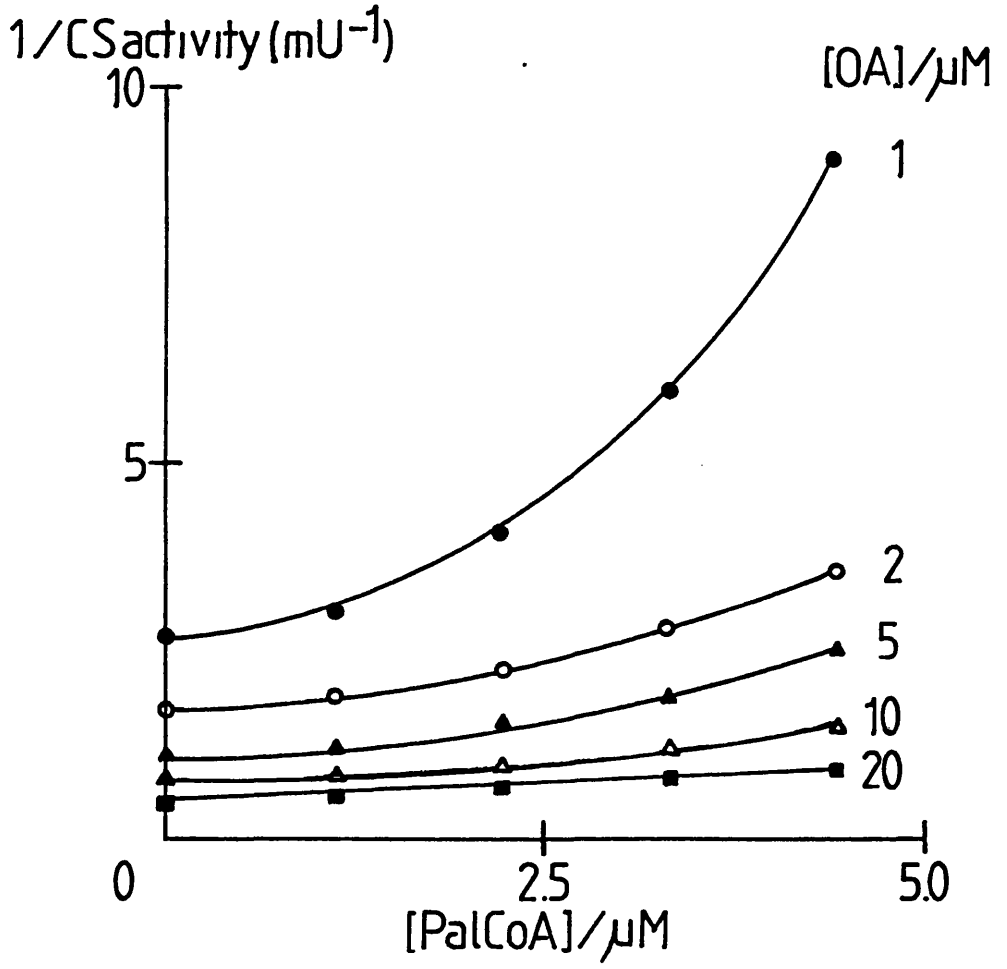
Dixon plots (Figs. 5.11 & 5.12) curved upwards

Figure 5.10: Inhibition ofBacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A: Effect of Different Order of Additions



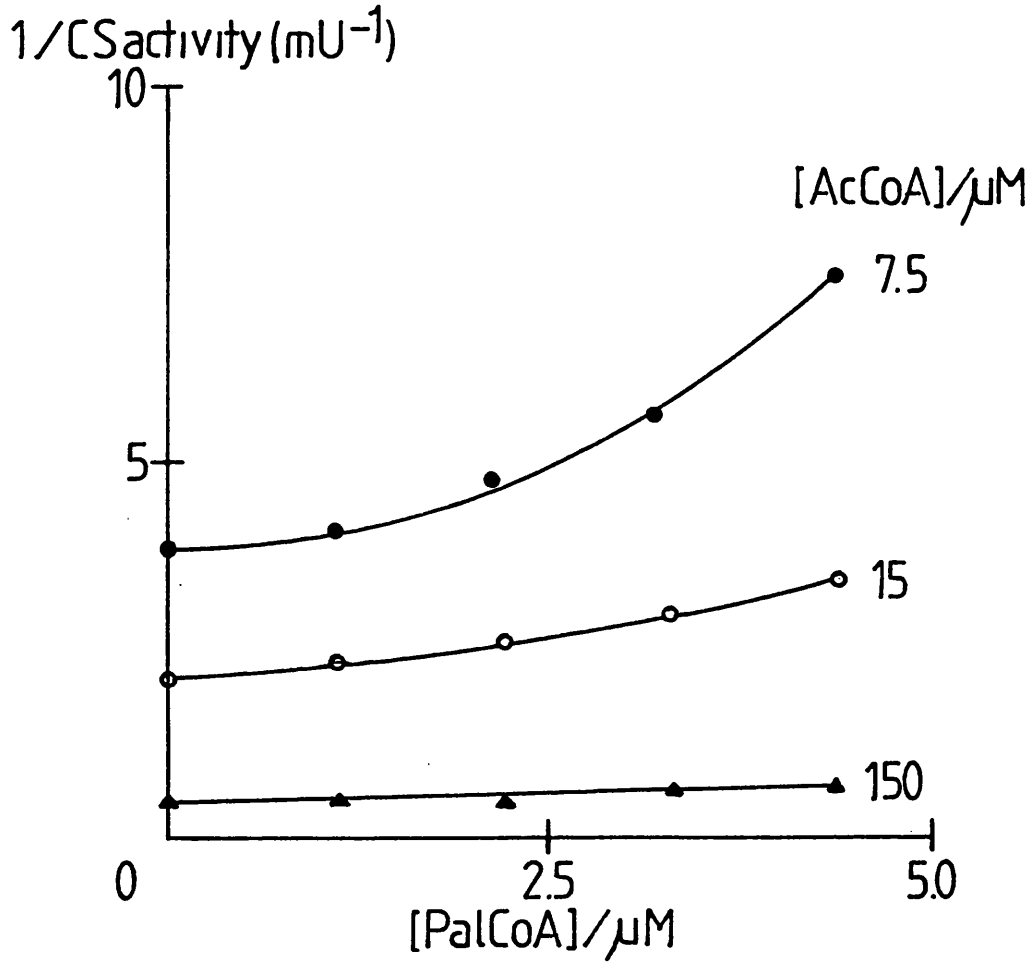
The assays were carried out as described in 3.4.1.

Figure 5.11: Dixon Plots of the Inhibition of Bacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A as Oxaloacetate Concentration is Varied



The data were plotted according to the method of Dixon (1953). The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

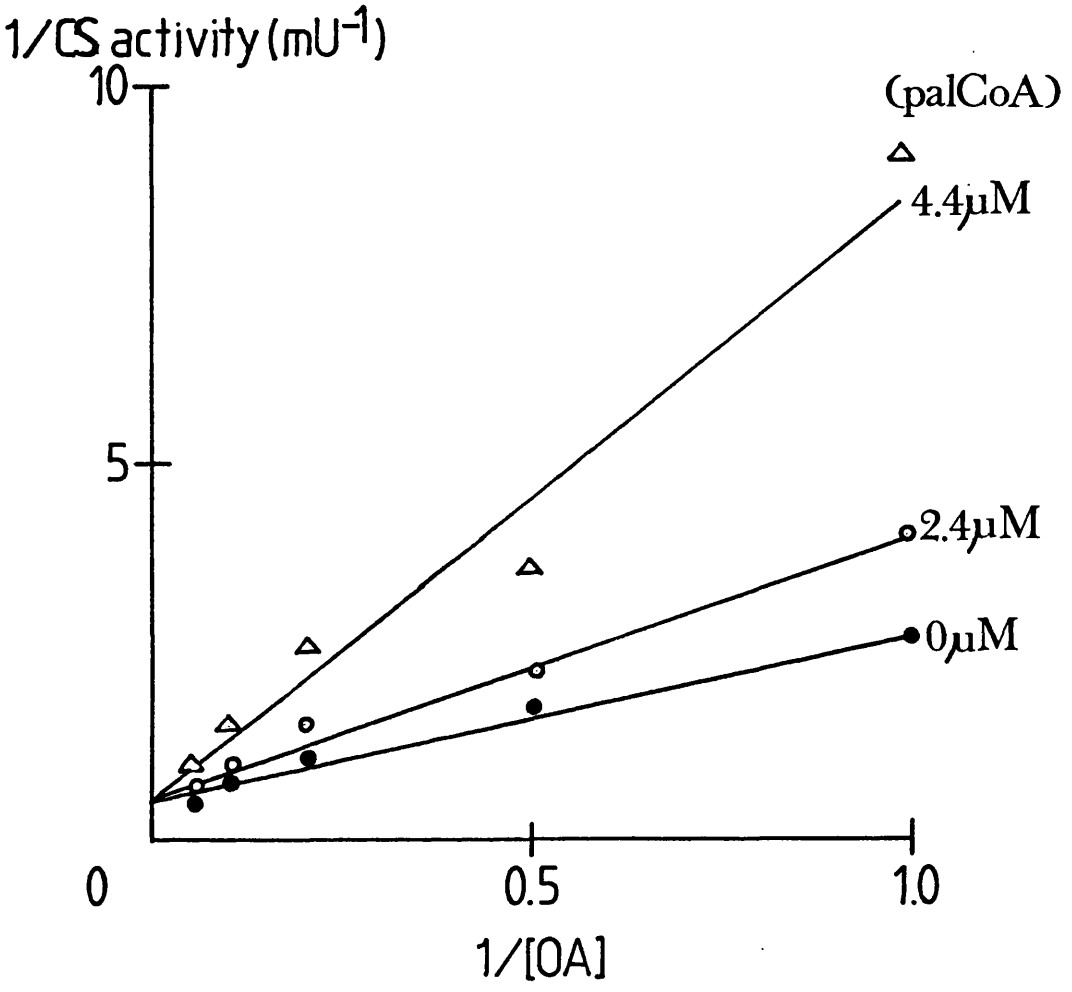
Figure 5.12: Dixon Plots of the Inhibition of Bacillus megaterium Citrate Synthase by Palmitoyl-coenzyme A as AcCoA Concentration is Varied



The data were plotted according to the method of Dixon (1953). The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

which suggests either co-operative inhibition or that more than one inhibitory effect is occurring. The curvature becomes more marked as either substrate is decreased; with substrate concentrations much above Km (9±3μM for OA; 88±38μM for AcCoA - Robinson *et al.*, 1983a) little curvature is apparent. Interestingly, with OA, a double reciprocal plot of activity v substrate concentration at various concentrations of palCoA (Fig. 5.13) gave the same result as expected from simple competitive inhibition. Wieland *et al.* (1964a) showed that a double reciprocal plot of activity of pig heart CS v OA concentration gave the competitive inhibition pattern. Srere (1965) pointed out that the effect of OA is best thought of in terms of protection of the enzyme, but from the competitive binding experiments of Caggiano & Powell (1979), it is clear that palCoA and OA do affect each other's binding. As we have seen, OA is also a powerful protector of *Bacillus megaterium* CS and in view of this, and of other similarities, it is probable that its effect on palCoA inhibition is the same as for pig heart CS. These experiments demonstrate the general unreliability of the double reciprocal plot, especially in detecting deviations from Michaelis-Menten kinetics. They also demonstrate the difficulty in assessing the nature of apparently competitive effects: from kinetic data only, one can never be certain whether the two compounds are competing for the same site or whether there are two different sites, one being blocked or

Figure 5.13: Double Reciprocal Plot of Activity of Bacillus megaterium Citrate Synthase against Oxaloacetate Concentration with Varying Concentrations of Palmitoyl-coenzyme A



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

destroyed by a conformational change caused by binding at the other.

5.2.3 Inhibition of Citrate Synthases by Palmitoyl-thioglycollate

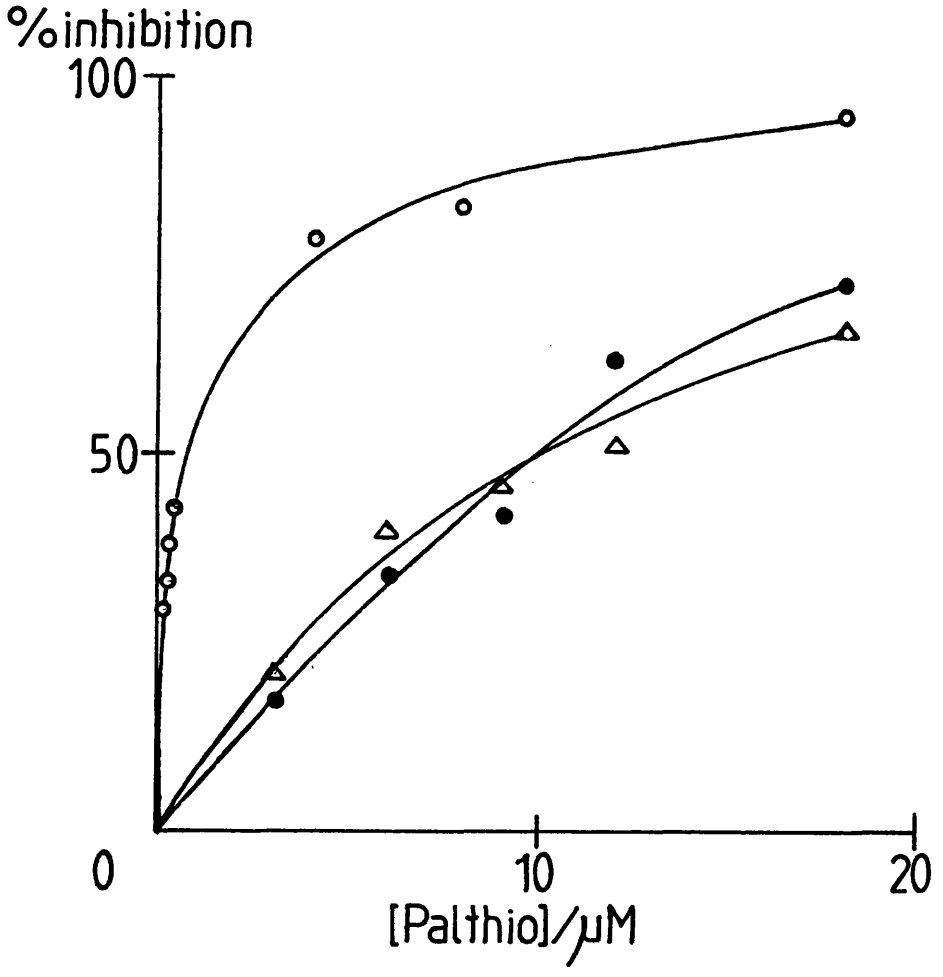
Palthio, an intermediate in the synthesis of palCoA (3.9), inhibited CS almost as powerfully as palCoA (Fig. 5.14); again, E.coli CS was the most sensitive, whilst the effects on Bacillus megaterium CS and pig heart CS were almost identical. Though the behaviour of the CSs in response to palthio was similar to that in response to palCoA, it did differ in the lack of time dependence (Fig. 5.15), though this may merely reflect a faster rate of binding. On dilution into the assay buffer (ET8), Bacillus megaterium CS was inactivated (presumably due to the lack of glycerol which is required to stabilize the enzyme)(Fig. 5.15); AcCoA and especially OA protected against this inactivation. Addition of palthio did not increase the rate of loss of activity nor did it prevent the protective action of AcCoA or OA.

5.2.4 Inhibition of E.coli Citrate Synthase by Palmitate

Palmitate had no effect on the CSs of Bacillus megaterium and pig heart at concentrations up to the solubility limit of the palmitate in aqueous solution (0.2mM). However, E.coli CS was inhibited (to a maximum of about 30%) by low concentrations of palmitate (Fig. 5.16). This may in part explain the greater sensitivity of E.coli CS to both palCoA and palthio.

5.2.5 Inhibition of Citrate Synthase by Ethanol

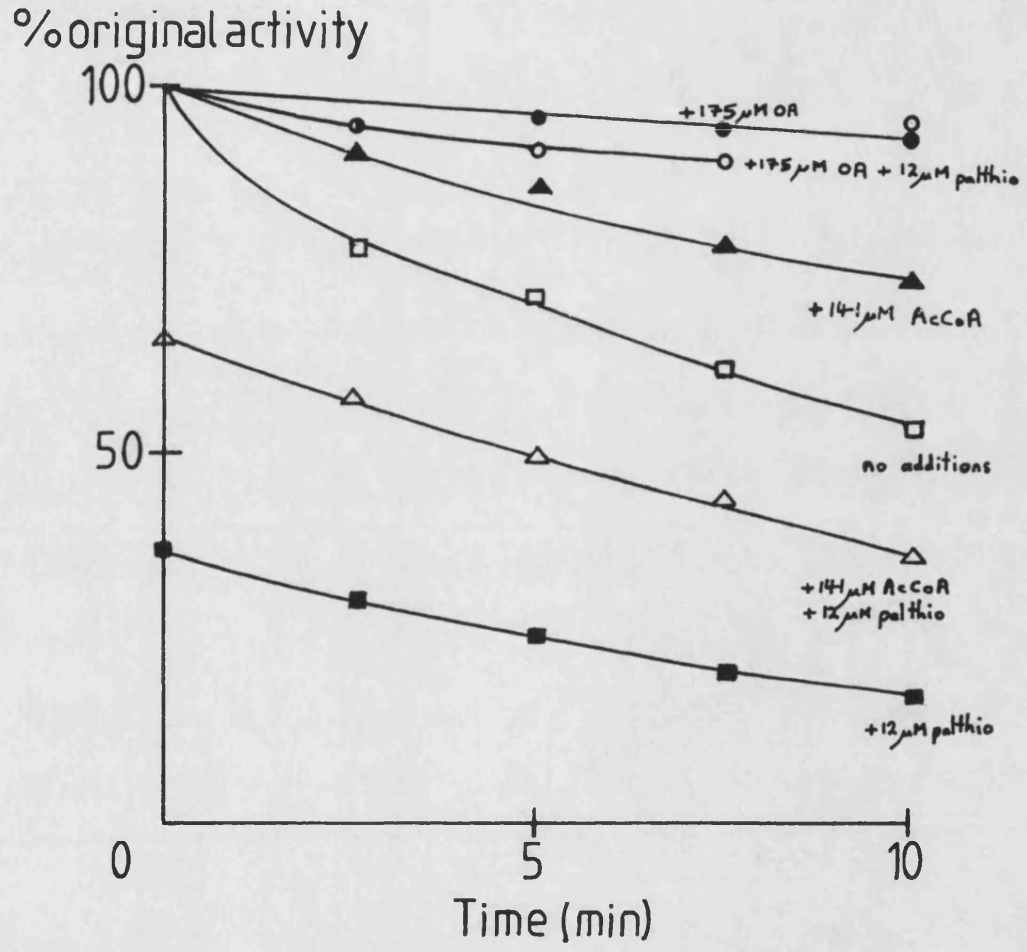
Figure 5.14: Inhibition of Citrate Synthases by Palmitoyl-thioglycollate



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction. ET8 buffer was used throughout.

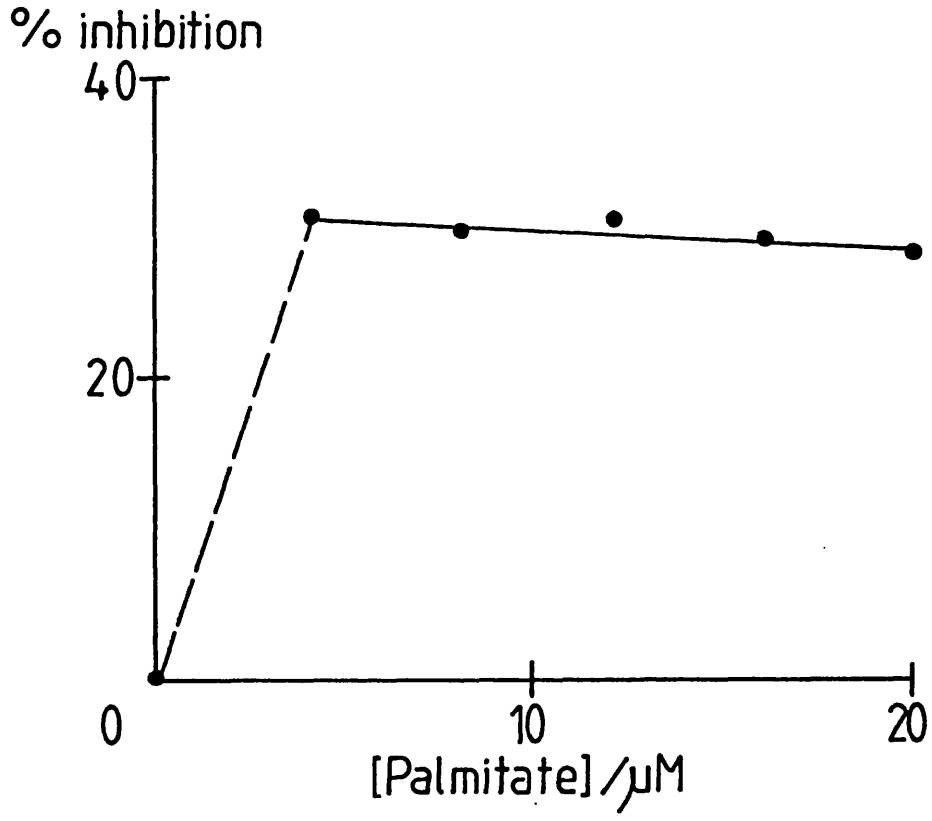
- ○ 7.9mU E.coli CS
- ● 7.2mU B.megaterium CS
- △ 9.2mU pig heart CS

Figure 5.15: Inactivation of Bacillus megaterium Citrate Synthase with Time in the Presence and Absence of Palmitoyl-thioglycollate showing the protective effects of Oxaloacetate and AcCoA



The assays were carried out as described in 3.4.1. Enzyme and inhibitor were added to the buffer along with the additions as indicated. The solution was incubated for the time indicated before the addition of AcCoA and/or OA as appropriate.

Figure 5.16: Inhibition of E.coli Citrate Synthase by Palmitate



The assays were carried out as described in 3.4.1, using ET8 buffer. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction.

Both palthio and palmitate are less water soluble than palCoA and had to be dissolved in ethanol. Ethanol was found to have slight inhibitory effects on all three CSs (Fig. 5.17); again, E.coli CS was the most sensitive. Inhibition by ethanol was taken account of in the experiments with palthio and palCoA.

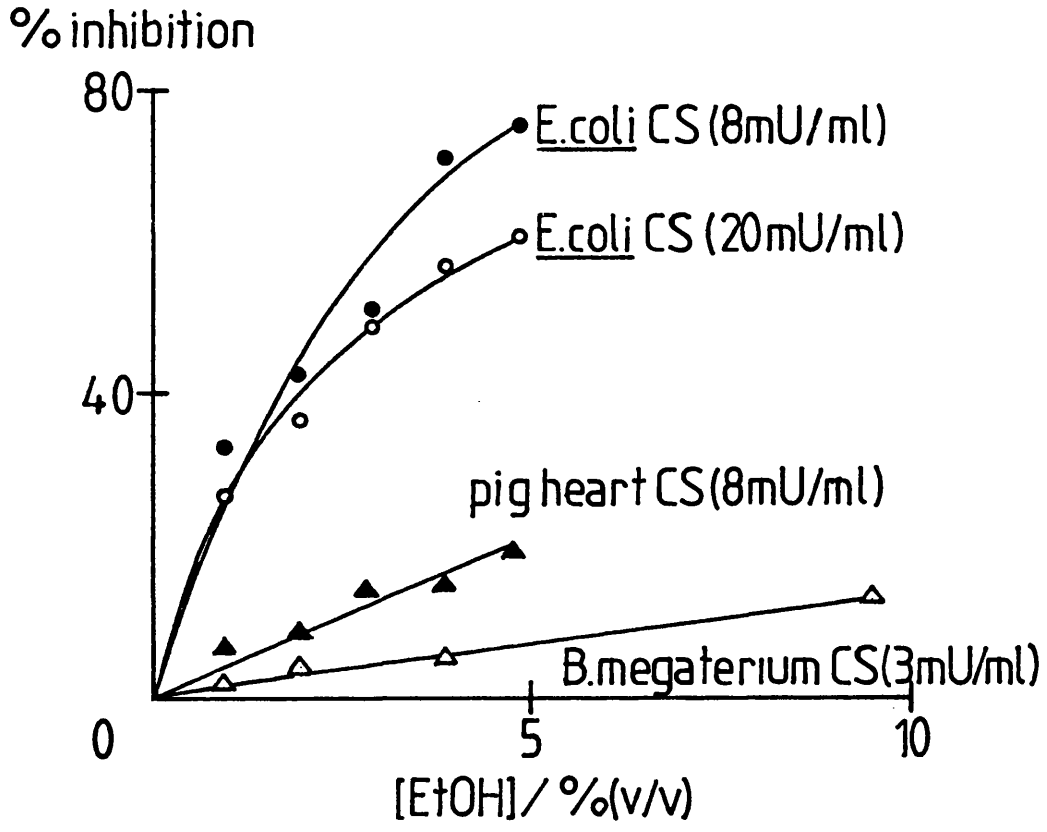
5.2.5 The Effect of Different Orders of Additions of Substrates, Enzymes, and Inhibitors to the Citrate Synthase Assay Mixture

As stated above (5.2.2) concerning the effect of palCoA on Bacillus megaterium CS, different rates are obtained when the components are added in different orders, presumably due to the protective action of OA and AcCoA. The same appears to be true for the effect of palthio on Bacillus megaterium CS (Fig. 5.15). Table 5.1 shows the results of a survey of the effect of the different orders of additions with palCoA, palthio, and palmitate, and with E.coli, Bacillus megaterium, and pig heart CSs. In all cases, the same phenomena were observed: OA conferred a large degree of protection, whilst AcCoA protected to a lesser extent.

5.2.6 Cleavage of the Thioester Bond of Palmitoyl-coenzyme A and Palmitoyl-thioglycollate Results in the Loss of Inhibitory Action

The thioester bond of palCoA can be cleaved by incubation for 90min at pH 13 and 50^{o}C. The thioester bond of palthio can be cleaved by mild heat. PalCoA and palthio treated in these ways only poorly inhibited the

Figure 5.17: Inhibition of Citrate Synthases by Ethanol



The assays were carried out as described in 3.4.1. There was no preincubation of enzyme and inhibitor. Enzyme was added to start the reaction. ET8 buffer was used throughout.

Table 5.1: The Effect of Different Orders of Additions of Substrates, Enzymes, and Inhibitors on Citrate Synthase Activity

Inhibitor	Reaction started with	E.coli	B.megaterium (mU per assay)	Pig Heart
none	OA	20.1	34.2	40.8
7.8μM	e	7.0		
palmitate	OA	6.3		
	AcCoA	7.9		
15.6μM	e	2.9		
palmitate	OA	3.1		
	AcCoA	4.4		
181μM	e		32.0	40.8
palmitate				
none	OA	10.1	3.0	6.0
47nM	e	4.8		
palCoA	OA	1.1		
	AcCoA	4.0		
12μM	e			5.3
palCoA	OA			0.2
	AcCoA			3.5
22μM	e		1.7	
palCoA	OA		0.1	
	AcCoA		0.9	
none	OA	8.4	7.2	9.2
2μM	e	5.2		
palthio	OA	1.2		
	AcCoA	4.3		
12μM	e		4.8	4.7
palthio	OA		2.7	0.8
	AcCoA		4.0	4.0

Assays were carried out as described in 3.4.1. ET8 buffer was used throughout.

CSs of Bacillus megaterium and pig heart; E.coli CS was inhibited by about 40% with cleaved palCoA and by about 35% with cleaved palthio, presumably due to the inhibitory effect of palmitate on this CS (Table 5.2).

In these experiments, cleavage of the thioester bonds resulted in the generation of free SH groups. The free SH groups react with DTNB, making the use of the DTNB assay impractical. The discontinuous assay using DNPH was designed for the purposes of this experiment (4.7).

5.2.7 The Greater Sensitivity of E.coli Citrate Synthase to Palmitoyl-coenzyme A and Palmitoyl-thioglycollate

Palmitate is known to bind to pig heart CS, but without causing an inhibitory effect (Srere, 1965); it presumably does the same with Bacillus megaterium CS - certainly there was no inhibition. Of the three CSs, the E.coli enzyme is uniquely sensitive and this partly explains its greater sensitivity to palCoA and palthio, although, as it only inhibits up to about 30%, it cannot explain all of the greater sensitivity. Moreover, if the greater sensitivity to palCoA and palthio is explained by sensitivity to palmitate, then the sensitivity to palmitate remains to be explained. As it is not capable of fully inhibiting E.coli CS, palmitate cannot cause any major disturbance to the structure of the enzyme. It may cause some conformational change that slightly alters the enzyme active site and so reduces its activity. Alternatively, binding of the palmitate may give rise to

Table 5.2: Relief of Inhibition by Palmitoyl-coenzyme A and Palmitoyl-thioglycollate on Breakage of the Thioester Bond

	CS (mU per assay)		
	E.coli	B.megaterium	Pig Heart
palCoA (300μM)			
-palCoA	1.64(100%)	1.50(100%)	1.92(100%)
+palCoA	<0.01(0%)	<0.01(0%)	0.01(0.5%)
+treated palCoA (thioester bond broken)	0.97(59%)	1.36(91%)	1.91(99.5%)
palthio (260μM)			
-palthio	1.64(100%)	1.50(100%)	1.92(100%)
+palthio	<0.01(0%)	0.13(9%)	0.08(4%)
+treated palthio (thioester bond broken)	1.07(65%)	1.39(93%)	1.66(86%)

The assays were carried out as described in 3.4.1. ET8 buffer was used throughout.

a highly hydrophobic region in the vicinity of the active site and this may discourage the entry and/or exit of the hydrophilic molecules taking part in the enzyme-catalyzed reaction (see also 5.2.8).

E.coli CS is also the most sensitive to ethanol. It may be that the greater sensitivity of E.coli CS to these compounds is a reflection of the more complex quaternary structure of the molecule as compared with dimeric CSs. Under certain conditions, the purified enzyme exists in a dynamic equilibrium of different multimeric forms (Wright & Sanwal, 1971); a disturbance of this equilibrium could be the cause of the greater sensitivity. It would therefore be worthwhile to look at the effects on Acinetobacter calcoaceticus CS, since this is hexameric but it does not exist in an equilibrium of different forms.

5.2.8 Palmitoyl-coenzyme A as a Anchor for Citrate Synthase

An alternative explanation for the interaction of CS with palCoA is that it is involved with anchoring the enzyme to a membrane and/or to other related proteins such as MDH and aspartate aminotransferase (EC 2.6.1.1)(Fahien & Kmiotek, 1983; Webster et al., 1980). This could be of some significance due to the doubts cast upon the involvement in fat metabolism, and the observation that several molecules of palCoA are bound per molecule of CS. However, it does not explain the inhibitory action of palCoA; one would not expect an

anchor to be an inhibitor. But it is possible that the bound palCoA affects the entry of substrate molecules to the active site *in vitro*, whilst *in vivo* the substrate is provided by channeling through a loosely-associated multi-enzyme complex of citric acid cycle and related enzymes. Such complexes are known to exist *in vitro* (Robinson & Srere,1985; S.J.Barnes, personal communication), but their existence *in vivo* is hard to prove. As stated above (5.1.5), experiments with spin-labelled analogues of fatty acyl-CoAs do indicate that the fatty acyl chain of the bound fatty acyl-CoA has a high degree of mobility thus allowing the putative interactions to be theoretically possible. Such a hypothesis would also explain the almost identical effects of palCoA on enzymes such as glutamate dehydrogenase that, though unrelated to fat metabolism, could be involved in multi-enzyme complexes.

5.2.9 *The Irreversible Nature of the Inhibition/Inactivation*

There is some controversy in the literature as to whether the effects of palCoA are reversible or not. Srere (1965) described the inhibition as irreversible and he claimed that the results of Wieland *et al*. (1964a) agree with this. However, in that paper Wieland *et al*. actually reported a reactivation of palCoA-inhibited CS by the addition of BSA. Bloxham *et al*. (1980) reported that palCoA inhibition is rapidly reversed upon dilution.

In the studies described here, palCoA acted in

an irreversible manner. However, if a high concentration of enzyme to palCoA was used (as in the work of both Bloxham et al. (1980) and Wieland et al. (1964a)) then over 95% of the activity was recovered upon dilution after incubation of enzyme and palCoA for 5min. This suggests that irreversibility is dependent upon multiple binding to the enzyme.

5.2.10 Some Tentative Conclusions

All the phenomena described can be explained if one assumes that palCoA interacts with CSs in more than one way. Several molecules of palmitate can bind per molecule of pig heart CS, but without causing inhibition; the multiple binding of palCoA to the molecule is probably at the same sites. Possibly this binding is to hydrophobic sites and is responsible for the attachment of the enzyme to a membrane or to other associated enzymes. As discussed in 5.2.2, the multiple binding could explain the time dependence of the reaction and the apparent effect of the molar ratio of enzyme:palCoA on the inhibition.

In addition, palCoA binds in such a way that binding of OA is prevented and thus the enzyme is inhibited. An RCO-S-R' structure appears to be necessary for this inhibition. The efficacy of inhibition increases with the chain length of R; succinyl-CoA and butyroyl-CoA do not inhibit, the order of effectiveness of the inhibition of pig heart CS of some long chain fatty acyl-CoAs is octanoyl-CoA < dodecoyl-CoA < oleoyl-CoA <

palmitoyl-CoA < stearoyl-CoA (Wieland & Weiss,1963; Wieland et al., 1964a). The binding of the fatty acyl-CoA to the enzyme appears to occur towards the CoA part of the molecule, since from the spin labelled analogues, it was deduced that the end of the fatty acyl chain distal to the CoA had a greater degree of movement than the end proximal to the CoA. The inhibition could be caused by steric hindrance of the OA binding site, by conformational changes induced by palCoA binding, or by both. An analogue of oleoyl-CoA, that had an altered CoA part of the molecule, was a poorer inhibitor by an order of magnitude, which suggests that the inhibition is caused by conformational changes rather than steric hindrance; CoA must fit better into the site than the larger analogue. Palthio has a much smaller R' and so can fit into the site. It is a slightly worse inhibitor, but the fact that it inhibits at all suggests that the RCO-S-CH_2C part of the molecule must have some affinity for the site.

Hansel & Powell (1984) have shown that, whilst short chain fatty acyl-CoAs bind at the AcCoA site and do not promote further binding, long chain fatty acyl-CoAs bind at the AcCoA site in such a way as to promote further binding of fatty acyl-CoA or fatty acids. This would explain the co-operativity observed. Also, the binding of palmitate observed by Srere (1965) was in the presence of CoA; Hansel & Powell (1984) suggested that the occupation of the CoA binding site is necessary for

binding of fatty acids.

Physiological rationales involving binding of CS to membranes or to other enzymes appear to be more attractive than those concerning fat metabolism. In this context, the inhibition of CS observed *in vitro* perhaps does not occur *in vivo* because of further conformational changes induced by the enzyme's environment or because of correct channeling of the substrate as suggested in 5.2.8.

Reminiscent of the division in behaviour with respect ATP and NADH, the dimeric CSs of *Bacillus megaterium* and pig heart behaved identically in this study, whilst the CS of *E.coli* proved to be much more sensitive to the various compounds tested. Whether this difference is merely a reflection of the more highly organised structure of the *E.coli* CS or whether it has some greater significance is not clear.

6. A MODEL FOR PREDICTING THE CORRELATION BETWEEN THE RATE OF LOSS OF NATIVE PROTEIN AND THE RATE OF LOSS OF ACTIVITY DURING PROTEOLYSIS OF AN ENZYME

6.1 Introduction

As well as providing primary sequence information, limited proteolysis of an enzyme or enzyme complex can yield detailed information about conformational movements, and domain and subunit functions and interactions. To this end, predictive models of the effects of proteolytic digestion can be an invaluable aid in the interpretation of the data. This chapter describes a simple model for predicting the correlation between the rate of loss of native protein (on SDS-PAGE) and the rate of loss of enzyme activity during proteolytic digestion of the enzyme. The model was applied to data obtained from trypsinolysis of *E.coli*, *Bacillus megaterium*, and pig heart CSs; the results are described in chapter 7.

6.2 Nature of the Model

The model is concerned with deciding what effect the proteolysis of one subunit of a multisubunit enzyme will have on the other subunits and on the activity of the whole enzyme. It is in fact not one model, but several. Data can then be compared to the different models, and the best-fitting model selected. It should be stated at the outset that the best-fitting model can only be taken as an indication of what actually occurs; models are not reality, and they are plagued by

assumptions and simplifications, all of which could be invalid.

Whilst Bacillus megaterium and pig heart CSs are dimers, E.coli CS is a hexamer. It would be very simple to prepare models for the proteolysis of dimeric enzymes, but the hexamer represents a more difficult problem. The approach taken will be to describe the model for the hexamer, and once this has been elucidated, it can easily be used to take account of dimers.

6.3 Initial Assumptions

We will consider a hexameric molecule that is made up of 6 identical subunits, a, b, c, d, e, and f, all of which can exist in 2 forms: native and proteolyzed (the proteolyzed form can have an infinite number of subforms). In all the figures, the native subunit is represented as O , and the proteolyzed subunit is represented as Ø . It is assumed that proteolyzed subunits can be separated from native subunits by SDS-PAGE, and so on a densitometer scan of a Coomasie-stained gel, the peak corresponding to the native subunit is assumed to represent only such subunits; its area can therefore be taken as a measure of the amount of native subunit. At this stage, no assumptions are made concerning the activity of a molecule containing a mixture of native and proteolyzed subunits. The second major assumption, which can perhaps be criticized more than the first, is that all of the subunits are equally prone to proteolysis, and that this

condition is not affected by proteolysis of neighbouring subunits. The consequences of the breakdown of this and of the other assumption will be considered briefly later (6.8).

6.4 The Number of Different Hexamer Forms

During the course of the proteolysis, the hexamer will be in one of seven different forms, A to G, depending upon the number of subunits in the molecule that have been proteolyzed (Fig. 6.1). If we consider the B form, i.e., where one of the 6 subunits has been proteolyzed, then there are 6 different ways (subforms) in which this form can be obtained: proteolysis of subunit a, proteolysis of subunit b, etc.. In general, for any of the forms A to G, the number of subforms can be obtained from the formula for the number of combinations of n objects taken r at a time,

$$C = n!/[r!(n-r)!]$$

where in this case, n = 6 and r = the number of proteolyzed subunits. So, for example, with 3 proteolyzed subunits per molecule there are $6!/[3!(6-3)!] = 20$ subforms.

6.5 The Relative Numbers of Specific Subforms

In a solution containing a total of m subunits, n subunits per molecule, there will be m/n molecules. If we assume that, in total, i subunits have been proteolyzed, then the number of different ways (combinations) in which this could have occurred is given by z, where

Figure 6.1: The Different Hexamer Forms

$$z = \frac{m!}{i!(m-i)!} \qquad (1)$$

If we consider a specific subunit, a, of one of the molecules, then the number of the combinations, z, in which a has been proteolyzed is given by y, where

$$y = \frac{(m-1)!}{(i-1)!(m-i)!} \qquad (2)$$

and the number in which subunits a and b of the same molecule have been proteolyzed is given by x, where

$$x = \frac{(m-2)!}{(i-2)!(m-i)!} \qquad (3)$$

and similarly for the numbers in which a ,b, and c have been proteolyzed, and for a, b, c, and d, a, b, c, d, and e, and a, b, c, d, e, and f, we have equations 4 to 7:-

$$w\,(abc) = \frac{(m-3)!}{(i-3)!(m-i)!} \qquad (4)$$

$$v\,(abcd) = \frac{(m-4)!}{(i-4)!(m-i)!} \qquad (5)$$

$$u\,(abcde) = \frac{(m-5)!}{(i-5)!(m-i)!} \qquad (6)$$

$$t\,(abcdef) = \frac{(m-6)!}{(i-6)!(m-i)!} \qquad (7)$$

6.6 The Relative Numbers and Probabilities of the Major Forms (A to G)

From equation (6), u consists of 2 components: the number in which a, b, c, d, and e are all proteolyzed, but f is not, and the number in which a, b,

c, d, e, and f are all proteolyzed. To obtain the first number alone, we must subtract the second, which is in fact equal to t from equation (7). That is ,

$$u' = u - t \qquad (8)$$

If we now consider v, to obtain the number of combinations in which only a, b, c, and d are proteolyzed, we must subtract the number in which a, b, c, d, and e are proteolyzed, the number in which a, b, c, d, and f are proteolyzed, and the number in which a, b, c, d, e, and f are proteolyzed. That is ,

$$v' = v - 2u' - t \qquad (9)$$

Similar analysis is required to determine w', x', and y', the coefficients of the equations obtained being those of Pascal's triangle.

$$w' = w-3v'-3u'-t \qquad (10)$$

$$x' = x-4w'-6v'-4u'-t \qquad (11)$$

$$y' = y-5x'-10w'-10v'-5u'-t \qquad (12)$$

Equations (7) to (12) provide us with the numbers of combinations of one subform of each of the major forms, A to G. To obtain the numbers of combinations for all the subforms, we can simply multiply by the number of subforms. The relative probabilities of each of the major forms are then obtained by dividing these by z. Thus,

$$G = t/z \qquad (13)$$

$$F = 6u'/z \qquad (14)$$

$$E = 15v'/z \qquad (15)$$

$$D = 20w'/z \qquad (16)$$

$$C = 15x'/z \qquad (17)$$

$$B = 6y'/z \quad (18)$$

$$A = 1-(B+C+D+E+F+G) \quad (19)$$

Where A to G are the relative probabilities of the forms A to G of Fig. 6.1. By using these equations, we can determine the probabilities of each of the major forms for any value of i.

6.7 Description of the Different Models

We are now in a position to make assumptions as to the activity of the various proteolyzed species and to determine the consequences. In all of the models described, Y, the relative area, is given by

$$Y = (6A+5B+4C+3D+2E+F)/m \quad (20)$$

or more simply, by

$$Y = (m-i)/m \quad (21)$$

And this should correspond to the area of the peak of the native subunit on a densitometer scan of a gel. X, the relative activity, corresponds to the enzyme activity. Both X and Y are relative values, referring to the original, unproteolyzed solution. The scales of the graphs in chapter 7 are given in percentage terms, i.e., % native subunit remaining (equivalent to Y x 100) v % original activity (equivalent to X x 100).

6.7.1 Model 1: Only Unproteolyzed Hexamers are Active

The first case to be considered is where only the completely unproteolyzed form, A, is enzymically active. This was considered to be the case with hexameric CSs. In this case,

$$X = A/(m/6) \quad (22)$$

A graph of Y v X is shown in Fig. 6.2, curve a.

6.7.2 Model 2: The Hexamer Consists of two Active Trimers

In this case, A will be 100% active, B will be 50% active, some subforms of C and D will be 50% active and others completely inactive, E, F, and G will be completely inactive. Therefore,

$$X = \frac{A+(B/2)+(6/15)(C/2)+(2/20)(D/2)}{(m/6)} \quad (23)$$

Y v X is shown in Fig. 6.2, curve b. This is the same model as that which would have been obtained if we had started with a trimeric molecule, and assumed that only unproteolyzed trimers were active.

6.7.3 Model 3: Any Trimeric Moiety is Active

In this model, rather than considering the hexamer as a dimer of trimers, it is assumed that a proteolyzed molecule will retain 50% of its activity as long as at least three unproteolyzed subunits are present. From considerations of symmetry, it is therefore a much less realistic model than 2.

$$X = \frac{A+(B/2)+(C/2)+(D/2)}{m/6} \quad (24)$$

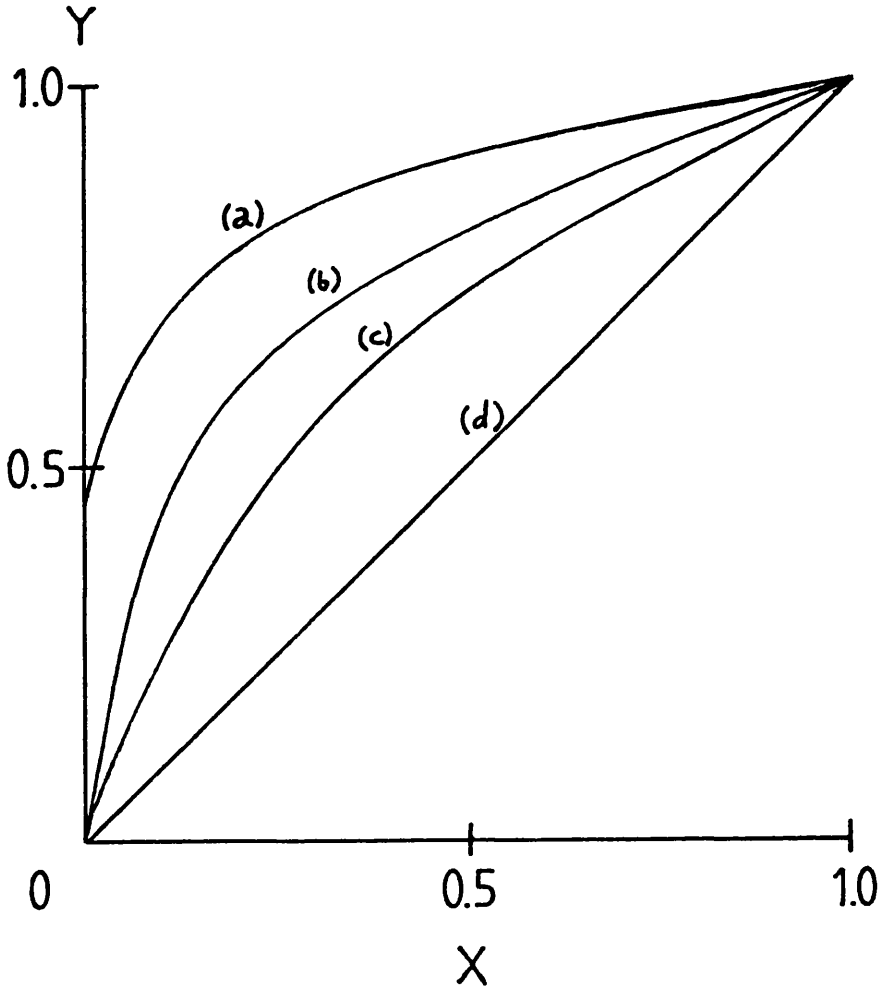
Y v X is shown in Fig. 6.3, curve b.

6.7.4 Model 4: The Hexamer Consists of Three Active Dimers

$$X = [A+(2/3)B+(3/15)(2C/3)+(12/15)(C/3)+(12/20)(D/3)+(3/15)(E/3)]/(m/6) \quad (25)$$

Y v X is shown in Fig. 6.2, curve c. This model is the same as that which would have been obtained if we had

Figure 6.2: Models of Proteolysis (A)



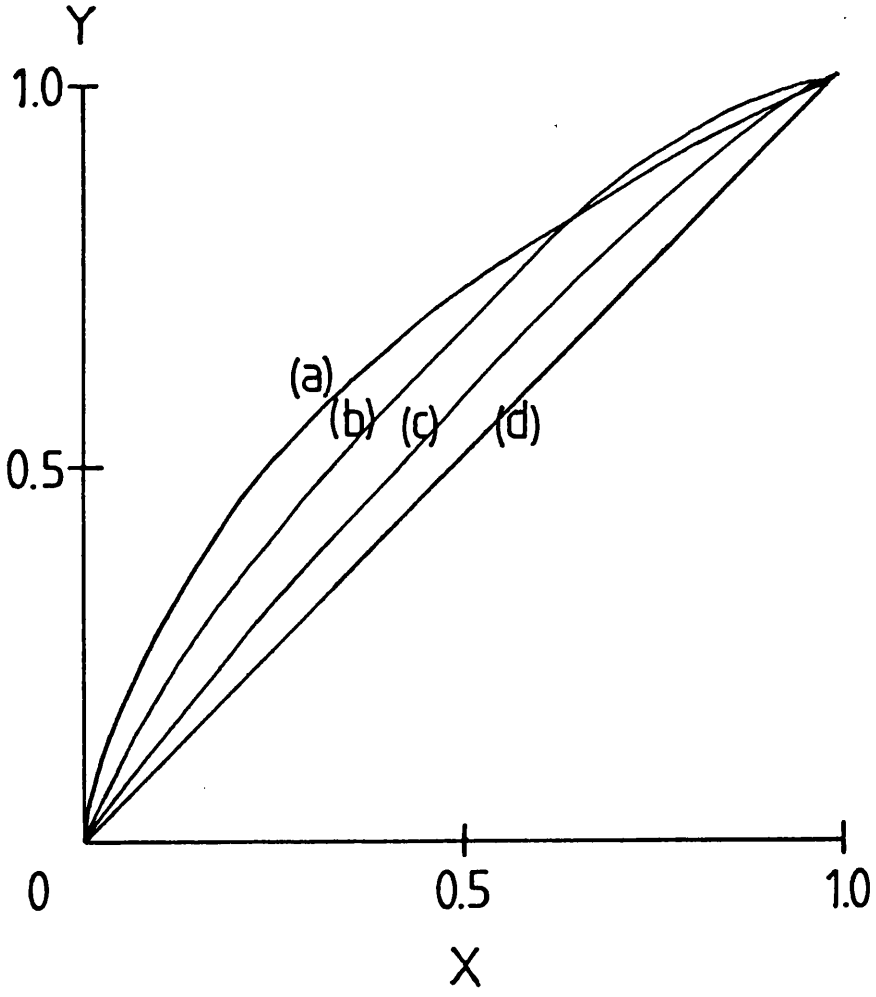
(a) Model 1: Only hexamers are active.

(b) Model 2: The hexamer consists of two active trimers.

(c) Model 4: The hexamer consists of three active dimers.

(d) Model 6: Any monomer is active.

Figure 6.3: Models of Proteolysis (B)



(a) Model 4: The hexamer consists of three active dimers.

(b) Model 3: Any trimer is active.

(c) Model 5: Any dimer is active.

(d) Model 6: Any monomer is active.

started with a dimeric rather than a hexameric molecule and had assumed that only unproteolyzed dimers were active. It can be shown that X and Y are related by the quadratic function

$$X = 2Y(Y.m-1)/m(m-1) \qquad (26)$$

6.7.5 Model 5: Any Dimeric Moiety is Active

This is a less realistic version than model 4

$$X = [A+(2/3)B+(2/3)C+(1/3)D+(1/3)E]/(m/6) \qquad (27)$$

Y v X is shown in Fig. 6.3, curve c.

6.7.6 Model 6: Any Monomeric Subunit is Active

This implies that proteolysis of a subunit does not at all affect the activity of the other subunits of the molecule.

$$X = [A+(5/6)B+(4/6)C+(3/6)D+(2/6)E+(1/6)F]/(m/6) \qquad (28)$$

Y v X is shown in Figs. 6.2 and 6.3, lines d. In this case, there is a simple linear relationship between Y and X, i.e., Y = X.

Models assuming that tetrameric or pentameric structures are the active forms need not be considered, since such structures do not satisfy symmetrical constraints.

6.8 Deviations from the Models

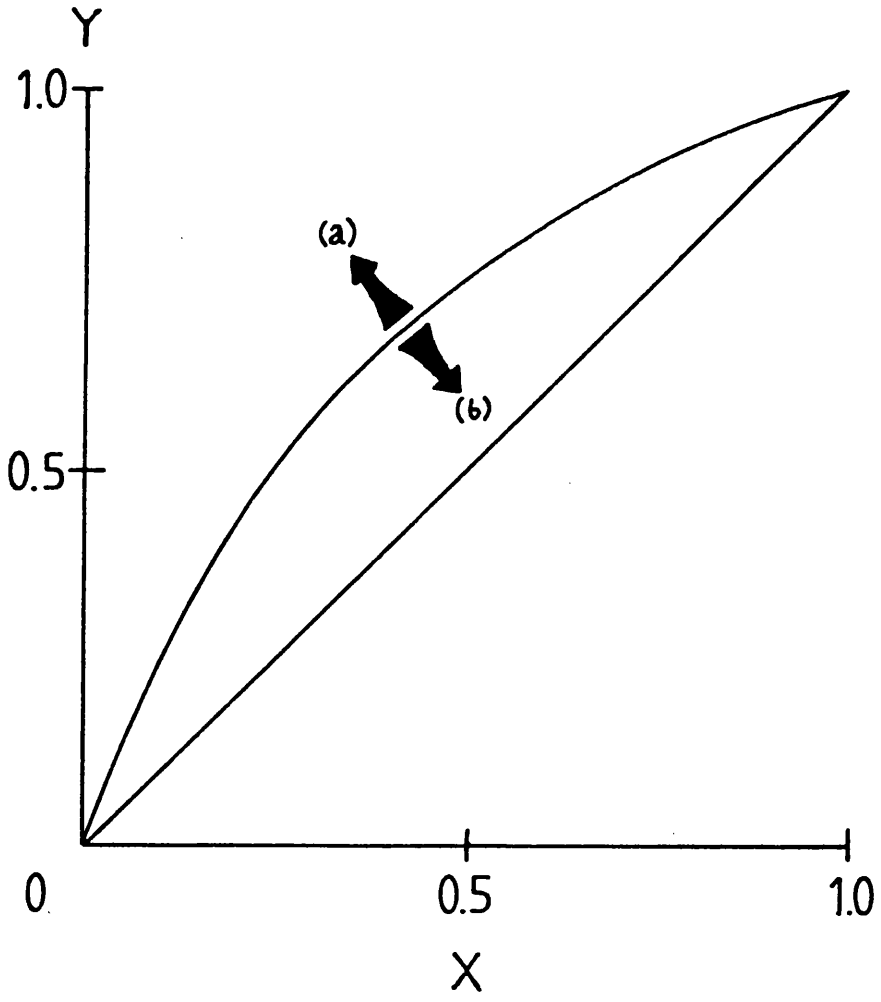
1. Proteolyzed subunits are not separated from native subunits on SDS-PAGE.

The effect of this would be to move the curves obtained upwards as shown in Fig. 6.4, arrow (a).

2. Proteolyzed subunits retain a fraction of activity.

This would move the curves downwards as

Figure 6.4: Deviations from the Models



(a) Upward displacement of the curves may be caused by lack of separation of proteolyzed from native subunits, or by differential susceptibility to proteolytic attack.

(b) Downward displacement of the curves may be caused by fractional activity of proteolyzed subunits, or by differential susceptibility to proteolytic attack.

indicated by Fig. 6.4, arrow (b)

3. Preferential attack of particular forms by the proteolytic enzyme.

If certain of the forms A to G are more susceptible to proteolytic attack than others, then the situation becomes far more complex. Precise analysis of all the possibilities would require computer fitting of the numerous parameters. However, in general terms, it can be seen that if proteolysis increases susceptiblity to further proteolysis then the curve will be shifted downwards, and vice versa. However, experimental data are not usually good enough to distinguish among all of the proposed models.

6.9 Application of the Model

6.9.1 Trypsinolysis of Citrate Synthases

In chapter 7, the data obtained from trypsinolysis of the CSs of E.coli, Bacillus megaterium, and pig heart are compared to the models described. As discussed there, in all cases except one, the data fit somewhere in between models 2 and 4. The deviations from the models are probably caused for the reasons described in 6.15. The models are not complete enough to determine exactly what is occurring, but they are sufficient to give a powerful indication that the active unit of both hexameric and dimeric CSs is the dimer.

6.9.2 Other Uses of this Method

This type of analysis can be applied to any situation in which there is a multimeric structure whose

components can exist in 2 (or more) forms. For example, it has been suggested that it could be applied to the problem of determining viable cell numbers of bacteria that exist in chains or other aggregates.

7. TRYPSINOLYSIS OF ESCHERICHIA COLI, BACILLUS MEGATERIUM, AND PIG HEART CITRATE SYNTHASES

7.1 Introduction

One of the simplest and most rewarding ways to obtain information about the structure of a protein is by proteolytic digestion. The proteolytic digest can be analyzed in a number of ways revealing different kinds of information.

Two major methods of analysis were used in this study: the assay of enzymic activity and the separation and quantitation of fragments by 10% SDS-PAGE. Only one proteolytic enzyme was used; different, but complementary information would be obtained from studies with other proteolytic enzymes. The model described in chapter 6 was applied to the data obtained. The emphasis was on a comparison of the behaviour of the different CSs.

7.2 The Trypsinolysis of Pig Heart Citrate Synthase

7.2.1 Previous Studies

The pig heart enzyme has already been substantially investigated by proteolysis, and such methods were used, for example, to determine the amino acid sequence (Wiegand et al., 1979; Bloxham et al., 1980, 1981, 1982; Bayer et al., 1981; Lill et al., 1984).

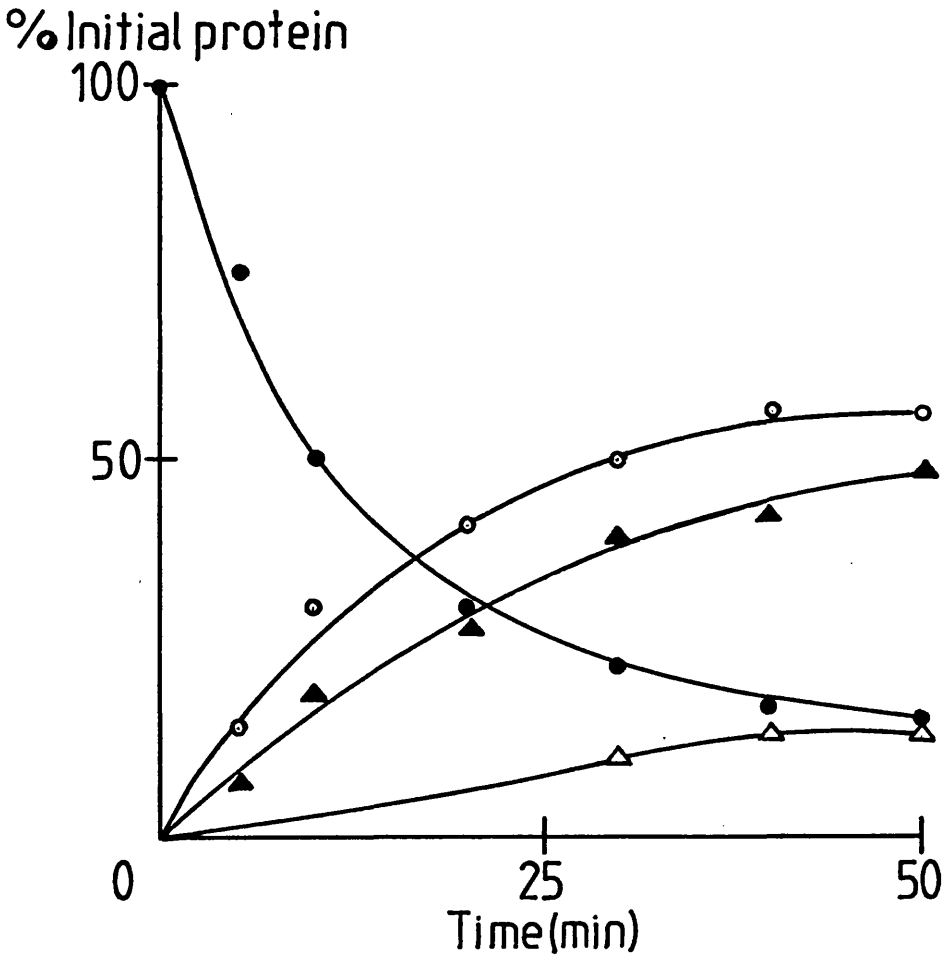
Trypsinolysis required the presence of palCoA. Bloxham et al. (1980) reported, but did not comment on, a

difference in the correlation of loss of enzyme activity with loss of native subunit between low (30 & 60 μM) and high (120 & 240μM) concentrations of palCoA. Regardless of the concentration of palCoA, initial trypsinolysis resulted in the cleavage of the monomeric subunit (Mr 45,000) into a large (Mr 35,000) and a small fragment (Mr 9,000). A secondary fragment of relative molecular mass 31,000 was produced, and the small fragment was substantially degraded by prolonged trypsinolysis.

7.2.2 Results

Very similar results to those described above were obtained in this study; the major fragments were of relative molecular mass 34,000 and 10,000, and the secondary fragment (Mr 30,000) was also observed. The rate of appearance of fragments is shown in Fig. 7.1. PalCoA was found to be necessary for trypsinolysis and was used at concentrations of 50μM and 150μM. No difference was seen in the relative molecular mass or the relative rates of appearance of the fragments produced, but a plot of percentage of native subunit remaining v percentage of original activity remaining (Fig. 7.2) was similar to that obtained by Bloxham et al. (1980) and shows a major difference in behaviour between the two concentrations of palCoA. Applying the model from chapter 6, it can be seen that, at the lower concentration of palCoA, the active unit of the CS appears to be the dimer, i.e., proteolysis of one subunit destroys the activity of the other subunit as well. However, at the higher concentration of palCoA,

Figure 7.1: Trypsinolysis of Pig Heart Citrate Synthase: The Rate of Appearance of Fragments



● native subunit

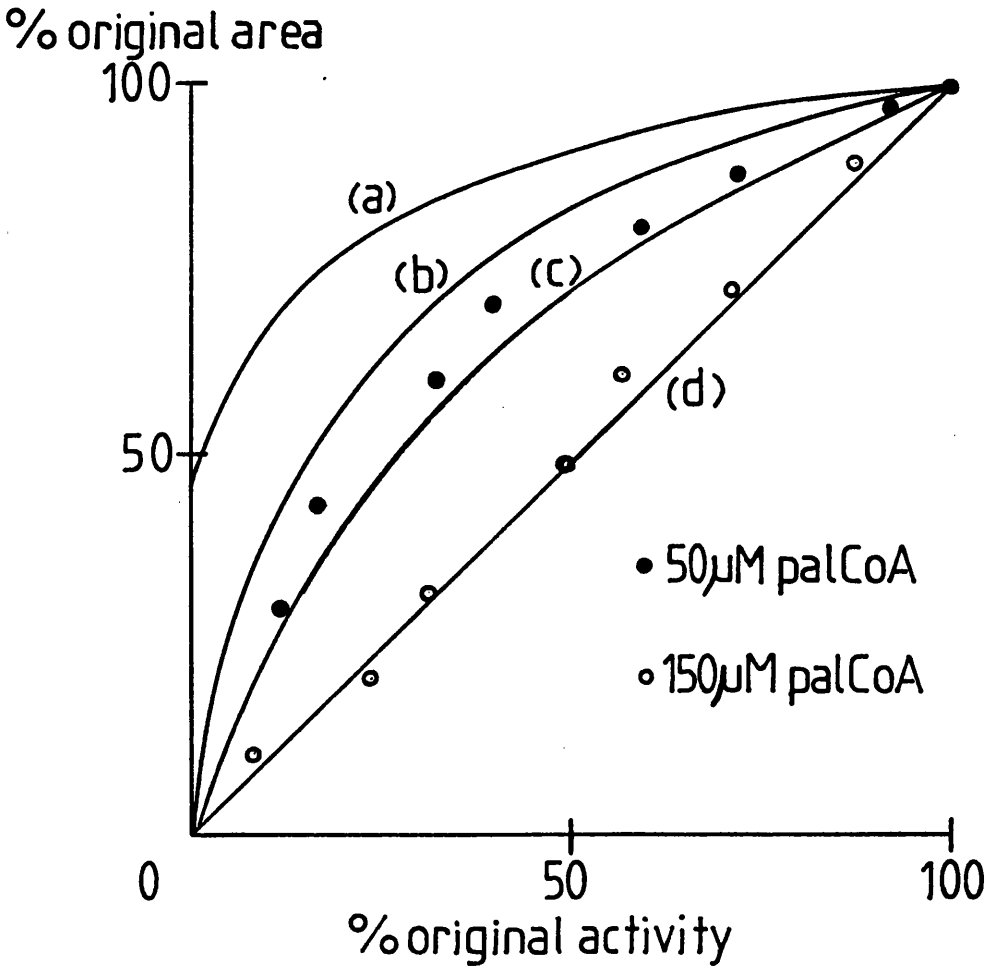
○ fragment, Mr 34,000

▲ fragment, Mr 10,000

△ fragment, Mr 30,000

The trypsinolysis was carried out as described in 3.20. CS was assayed as described in 3.4.1. 10%SDSPAGE was as described in 3.13.

Figure 7.2: Trypsinolysis of Pig Heart Citrate Synthase



The experimental data is compared with:-

(a) Model 1: Only hexamers are active.

(b) Model 2: The hexamer consists of two active trimers.

(c) Model 4: The hexamer consists of three active dimers.

(d) Model 6: Any monomer is active.

the enzyme behaves as though the active unit was the monomer, i.e., after proteolysis of one subunit, the other subunit retains its full activity. The enzyme is known to undergo conformational changes of the individual subunits (Bayer et al., 1981; Remington et al., 1982). These results suggest that there is a high degree of interaction between the subunits as well. As we have already seen, palCoA has a number of unexplained effects on CSs (chapter 5) including the promotion of interactions between CS and other proteins. Perhaps related to this is its ability to allow the activity of one subunit to be unaffected by the proteolysis of the other. There are two ways in which this could happen. Firstly, the palCoA could stabilize the partially proteolyzed enzyme, allowing the various conformational movements whilst preventing the total break-up of the structure. Secondly, the subunits may dissociate into inactive monomers; palCoA could then act by promoting the association of unproteolyzed inactive monmers into active dimers. The second possibility, though not totally ruled out, is unlikely since there is no evidence of dimer-monomer dissociation. Non-denaturing gel electrophoresis does not produce clear-cut answers because of the difficulty of quantitation of protein in the bands, and because the lack of a linear relationship between relative molecular mass and mobility leads to difficulties in interpretation. Any attempt to study dimer-monomer dissociation or association needs to be

done in the same buffer as the assay, if the two methods of observation are to be related.

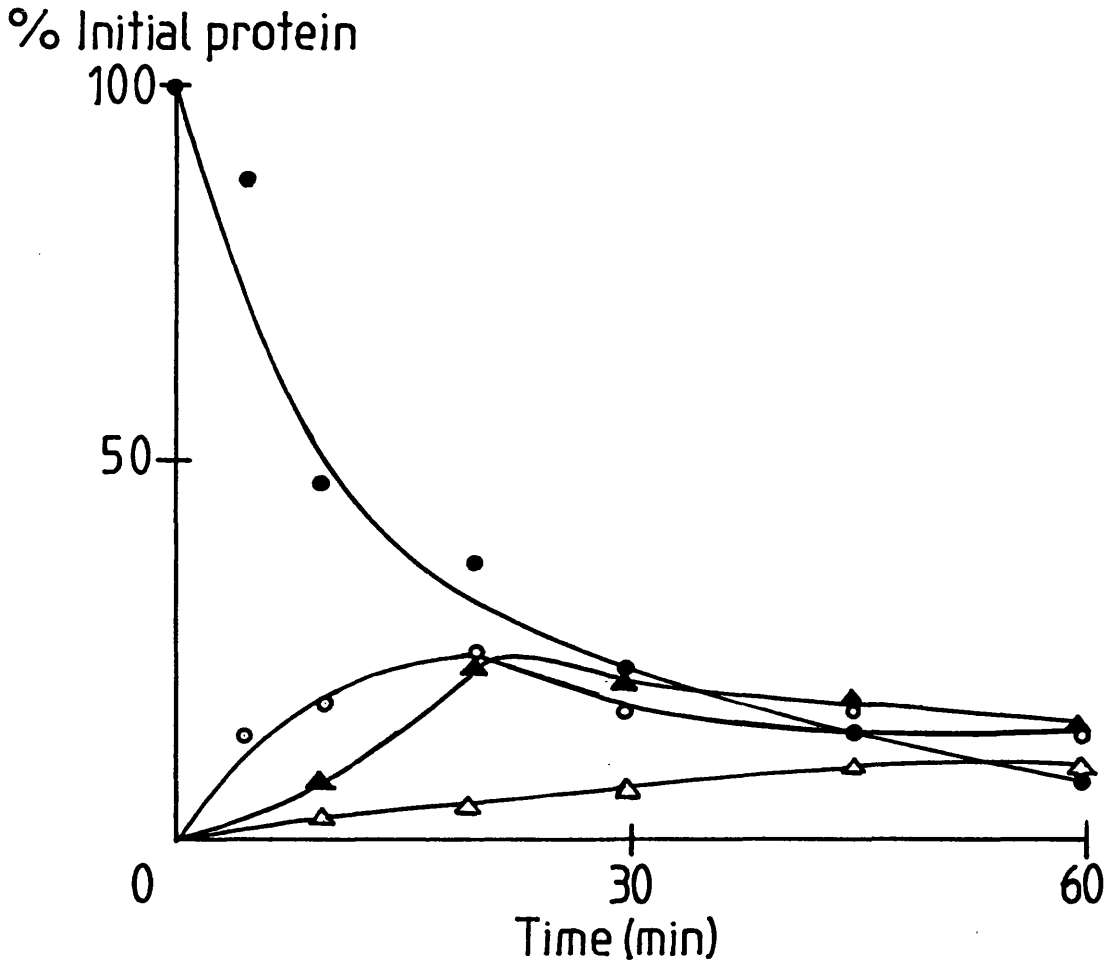
7.3 Trypsinolysis of Bacillus megaterium Citrate Synthase

Bacillus megaterium CS has only recently been purified, and this was the first proteolytic study of the enzyme. Three main tryptic peptides were produced: a large fragment (Mr 41,000), only just resolvable from the native subunit(Mr 42,000), and fragments of 28,000 and 27,000 (Fig. 7.3). The rate of production of these fragments is shown in Fig. 7.4; the large fragment appears first, followed by the 28,000 fragment, and then the 27,000 fragment. If a fragment of 28,000 is produced, then one would also expect a fragment of relative molecular mass approximately 14,000, but no such fragment was observed. Faint bands of relative molecular masses 22,000, 21,500, and 16,500 could be observed if an excess of proteolyzed protein was applied to the gels. Presumably the smaller fragments expected are rapidly digested to small peptides which are not detected.

The plot of percentage native subunit v percentage original activity gave the pattern expected with the dimer as the active unit (Fig. 7.4).

The trypsinolysis was performed in the presence and absence of 150μM palCoA; unlike pig heart CS, palCoA is not required for trypsinolysis. The palCoA did not increase the rate of trypsinolysis, and it did not noticeably affect the qualitative or quantitative results

Figure 7.3: Trypsinolysis of Bacillus megaterium Citrate Synthase: Rate of Appearance of Fragments



● native subunit

○ fragment, Mr 41,000

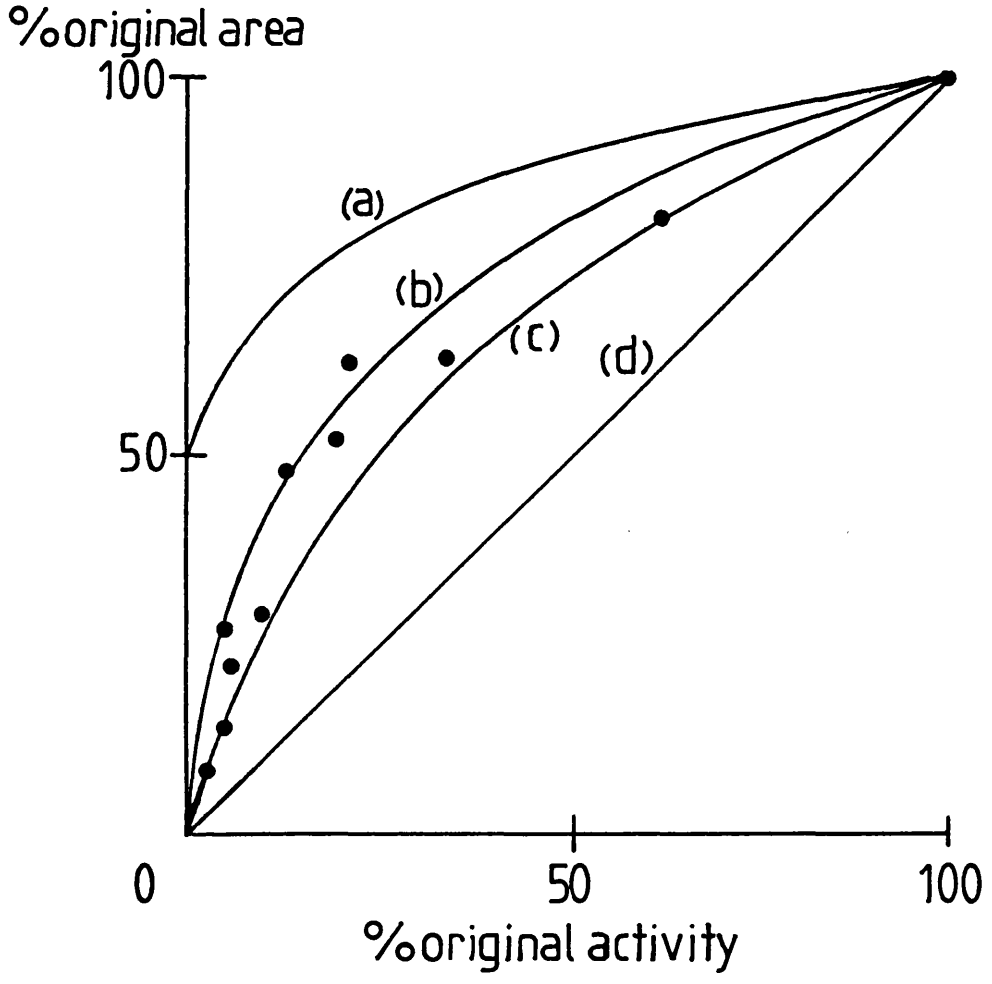
▲ fragment, Mr 28,000

△ fragment, Mr 27,000

The trypsinolysis was carried out as described in 3.20. CS was assayed as described in 3.4.1. 10%SDSPAGE was as described in 3.13.

N.B. See photo in back cover

Figure 7.4: Trypsinolysis of Bacillus megaterium Citrate Synthase



The experimental data is compared with:-

(a) Model 1: Only hexamers are active.

(b) Model 2: The hexamer consists of two active trimers.

(c) Model 4: The hexamer consists of three active dimers.

(d) Model 6: Any monomer is active.

obtained. Thus despite the similarity of behaviour of Bacillus megaterium and pig heart CSs with regard to palCoA inhibition, in this case there is a significant difference in the behaviour of the enzymes with respect to this molecule. However, it must be borne in mind that the Bacillus megaterium CS is inherently less stable than the pig heart enzyme: it requires the presence of 20%(v/v) glycerol to stabilize it; also it is significantly smaller than the pig heart enzyme (subunit Mr is 42,000 for Bacillus megaterium and 48,000 for pig heart). They are clearly different molecules, and even if they have very similar 3D structures and kinetic properties, differences in amino acid sequences are likely to produce differences in proteolytic patterns because of differences of susceptibility of the relevant residues (i.e., lys & arg in the case of trypsin). Presumably, in the pig heart enzyme, such residues are buried inside the molecule; the palCoA causes conformational changes that expose these residues. With the Bacillus megaterium enzyme, suitable sites for trypsin must be available on the surface of the molecule, and so proteolysis can occur without conformational changes.

7.4 Trypsinolysis of E.coli Citrate Synthase

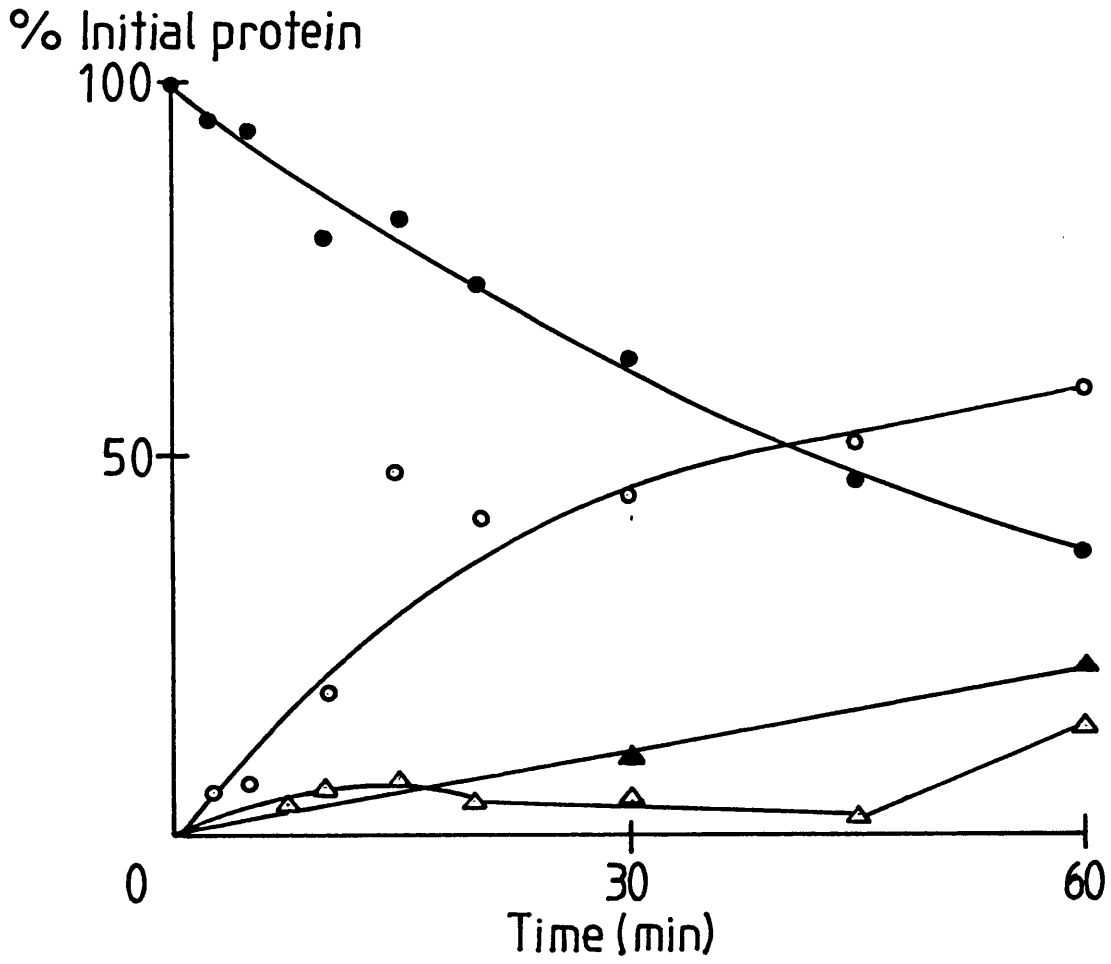
Bell et al. (1983) reported that trypsinolysis of E.coli CS produced a large fragment (Mr 32,000) and a small fragment (Mr 13,000); thus, except for not

requiring palCoA, the pattern of digestion was very similar to that obtained with pig heart CS. Similar results were obtained in this study: fragments of relative molecular masses 31,500 and 14,500 were obtained; in addition a third fragment of relative molecular mass 11,500 was obtained which appeared after the other two. The rate of appearance of fragments is shown in Fig. 7.5.

The plot of percentage native subunit v percentage original activity (Fig. 7.6) gave a pattern in between those expected for a dimer and a trimer. Comparison of these results with those from Bacillus megaterium CS (Fig. 7.7) show that the two enzymes behave in the same way; pig heart CS, at relatively low concentrations of palCoA, also behaves in the same way. With Bacillus megaterium and pig heart CSs the trimer may be ruled out as the active unit since the molecules are only dimers. With E.coli CS, whilst the trimer and even the hexamer as the basic active unit cannot be ruled out by this experiment, it seems likely that the basic active unit of this CS is also a dimer; the hexameric enzyme being a trimer of dimers.

There are three possible arrangements of the subunits in a hexameric protein (Fig. 7.8). Interacting domains are labelled p, q, r, and s according to the nomenclature of Cornish-Bowden & Koshland (1971). A trimer of dimers would give a trigonal prism, a dimer of trimers would give either an octahedral structure or a

Figure 7.5: Trypsinolysis of E.coli Citrate Synthase: Rate of Appearance of Fragments



● native subunit

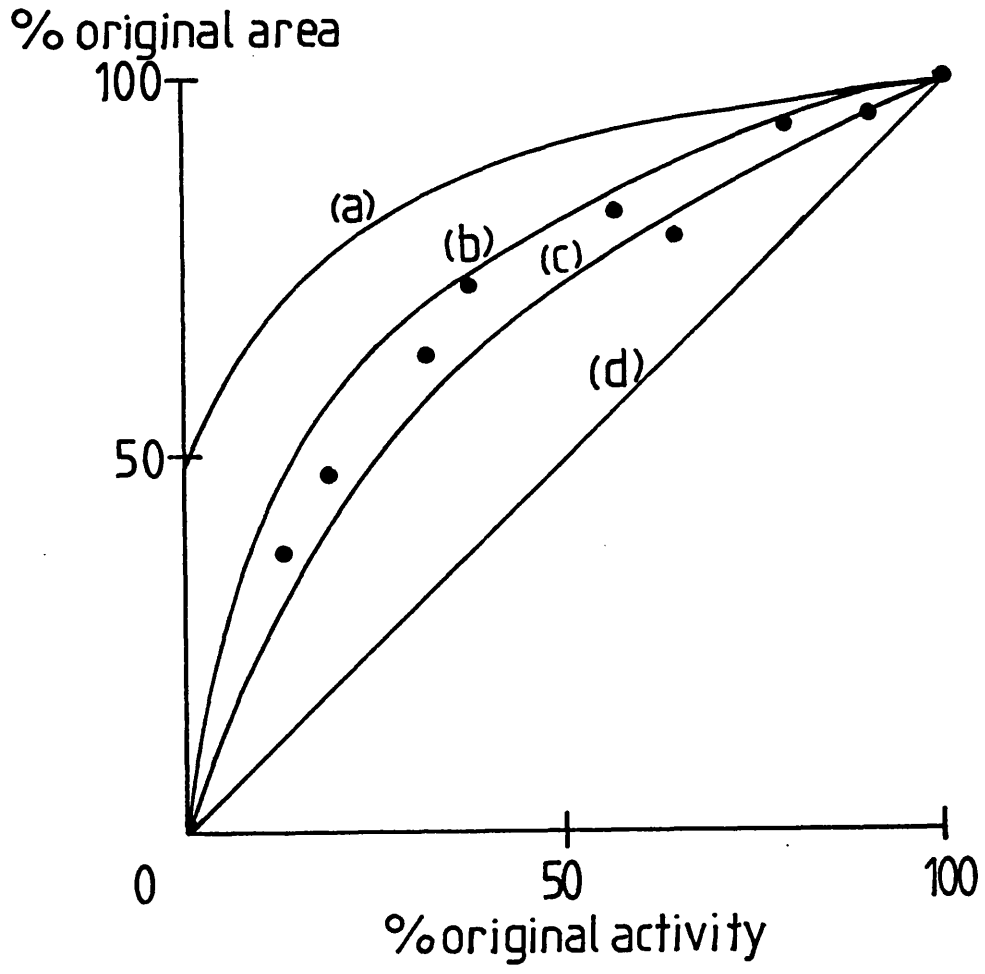
○ fragment, Mr 31,500

▲ fragment, Mr 14,500

△ fragment, Mr 11,500

The trypsinolysis was carried out as described in 3.20. CS was assayed as described in 3.4.1. 10%SDSPAGE was as described in 3.13.

Figure 7.6: Trypsinolysis of E.coli Citrate Synthase



The experimental data is compared with:-

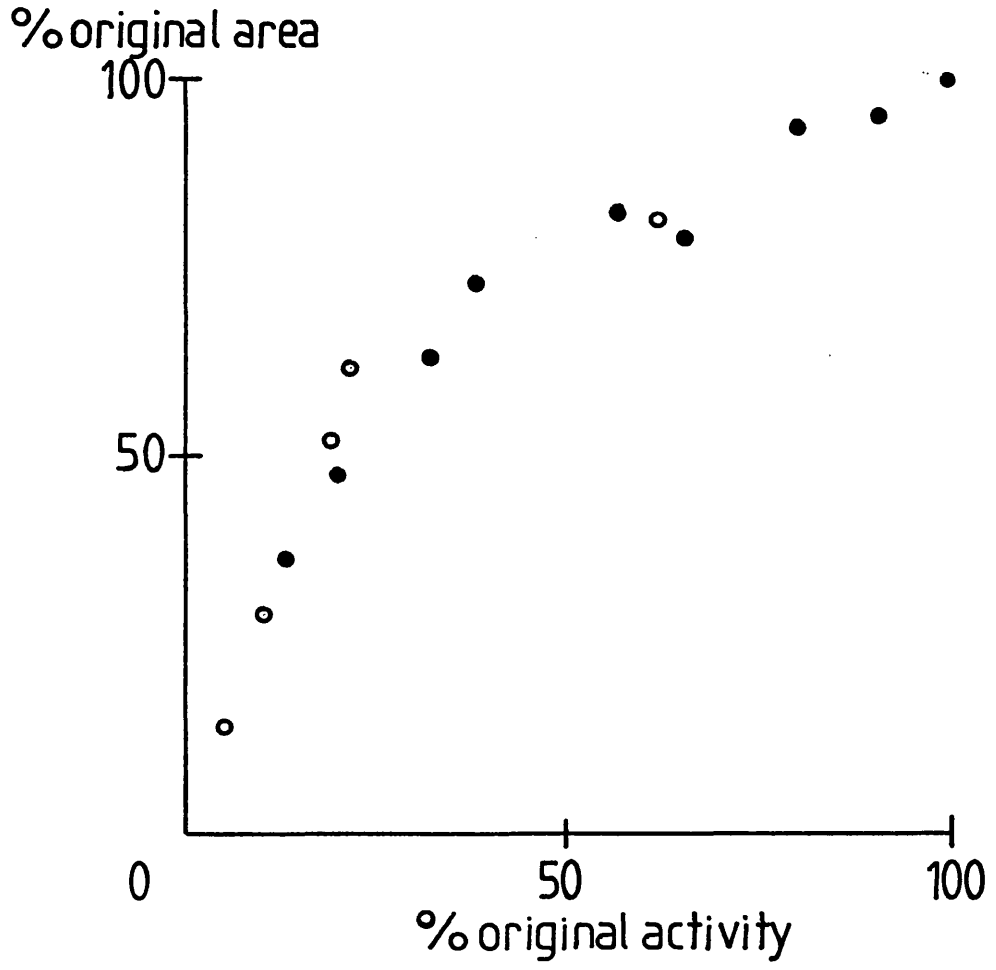
(a) Model 1: Only hexamers are active.

(b) Model 2: The hexamer consists of two active trimers.

(c) Model 4: The hexamer consists of three active dimers.

(d) Model 6: Any monomer is active.

Figure 7.7: Comparison of the Patterns of Trypsinolysis



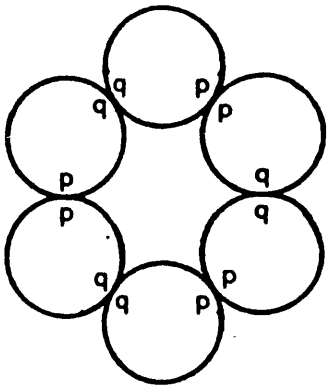
The data from the trypsinolysis of B.megaterium, and E.coli CSs are compared.

• E.coli

∘ B.megaterium

Figure 7.8: Possible Structural Arrangements of E.coli Citrate Synthase

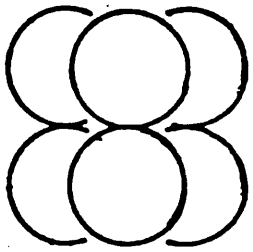
A



3 p–p ; 3q–q

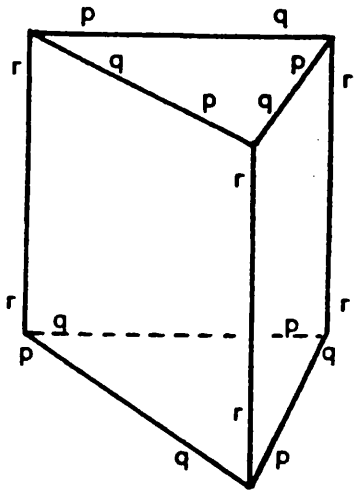
Planar Hexagon

B

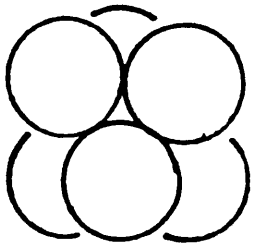


6 p–q; 3 r–r

Triganol Prism

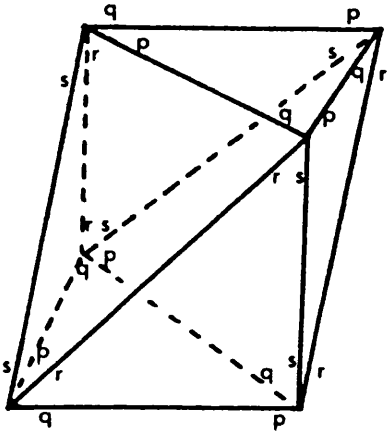


C



6 p–q ; 3 r–r ; 3 s–s

Octahedron



The merits of each arrangement are discussed in the text.

trigonal prism, and a hexamer could have any of the three structures. The planar hexagon structure for hexameric CSs can be eliminated in view of electron microscopy studies of CS from both E.coli (Danson, 1974) and Acinetobacter calcoaceticus (Rowe & Weitzman, 1969). Both of the other structures are consistent with electron microscopy, cross-linking studies (Robinson et al., 1983a), and with the results described here. However, as stated above, on the basis of analogy with Bacillus megaterium and pig heart CSs, the favoured model is the trimer of dimers and therefore the the trigonal prism ? structure. High resolution X-ray crystallography should help to solve this problem, as could other studies of the type described here but with different proteolytic enzymes.

The effect of palCoA on the trypsinolysis of E.coli CS as judged by enzyme activity could not be determined because the concentration of palCoA that was needed to cause a change in behaviour with pig heart CS caused complete inhibition of the more sensitive E.coli enzyme. However, palCoA did not affect the rate of trypsinolysis as judged by the decrease in amount of native subunit, and it did not affect the pattern of fragments obtained.

NADH inhibition of E.coli CS was followed throughout the course of trypsinolysis. It was found to be completely unaffected by the proteolysis.

7.5 Further Discussion

Differences in the size of fragments obtained on proteolysis of a family of related enzymes would be expected: sites for the proteolytic enzyme can be created or destroyed during evolutionary divergence from the 'ancestor' protein that could have little effect on the 3D structure of the molecule or on its activity. Thus structural differences will be observed that are not correlated with differences in activity. Therefore, despite the differences in the size of the fragments produced, the pattern of proteolysis of the three different enzymes is very similar when the loss of native subunit is compared with the loss of activity. The one notable difference here is the effect of palCoA on the pig heart enzyme; as already indicated (7.3), this difference could also be explained by the occurrence of functionally unimportant differences in amino acid sequence that result in different sites for proteolysis. The data strongly suggest that the active unit of all of the three CSs examined is a dimer.

It can be seen that structure-function relationships can be probed by proteolytic methods, and that models like the one presented in chapter 6 can aid in the analysis and indeed provide information that could not otherwise be obtained.

8. GENE CLONING EXPERIMENTS

8.1 Attempts to Clone the Gene from the Mutant Escherichia coli

8.1.1 Reasons for Wanting to Clone the Gene

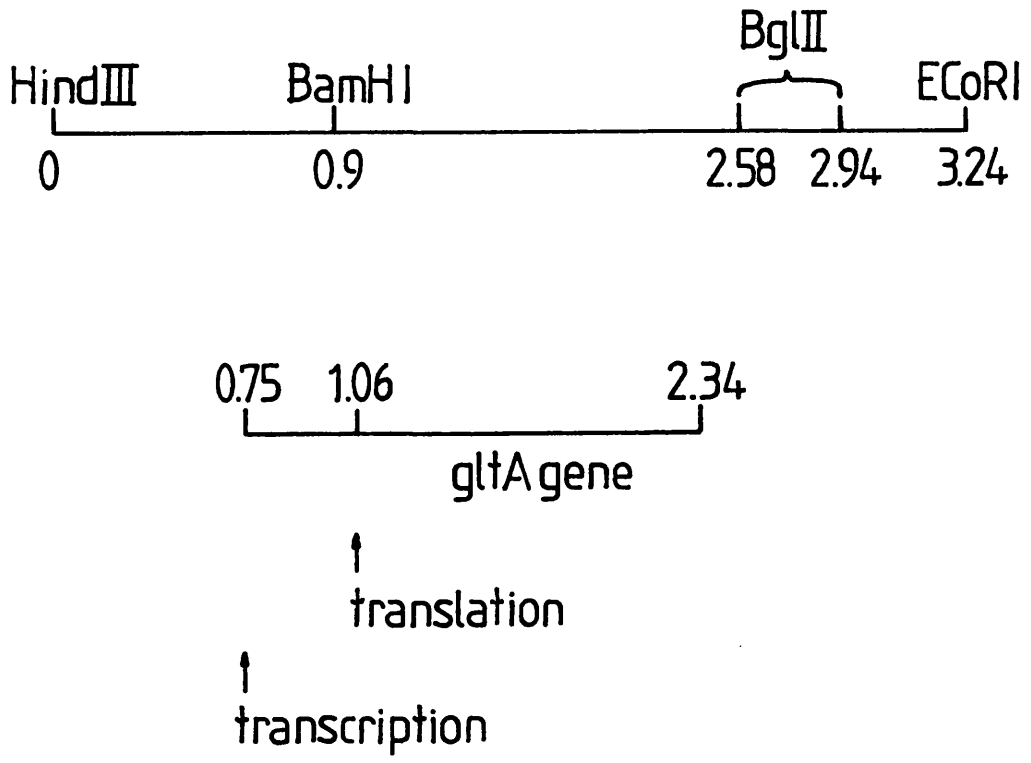
The E.coli mutant, Kllr3, with a small-type CS, is a relatively slow growing strain which gives poor yields of the mutant CS. This mutant CS is also less stable than the wild type protein. These factors combine to make it difficult to obtain pure mutant CS, but even so , it has been purified to a stage where only 2 bands are observed upon SDS-PAGE (Robinson, 1984).

By cloning the gene for the mutant enzyme, it may be possible to obtain amplified levels of the mutant CS and so facilitate purification. At the same time, the primary structure of the protein may be elucidated by the methods of recombinant DNA technology and gene sequencing.

8.1.2 Basis of the Cloning Strategy

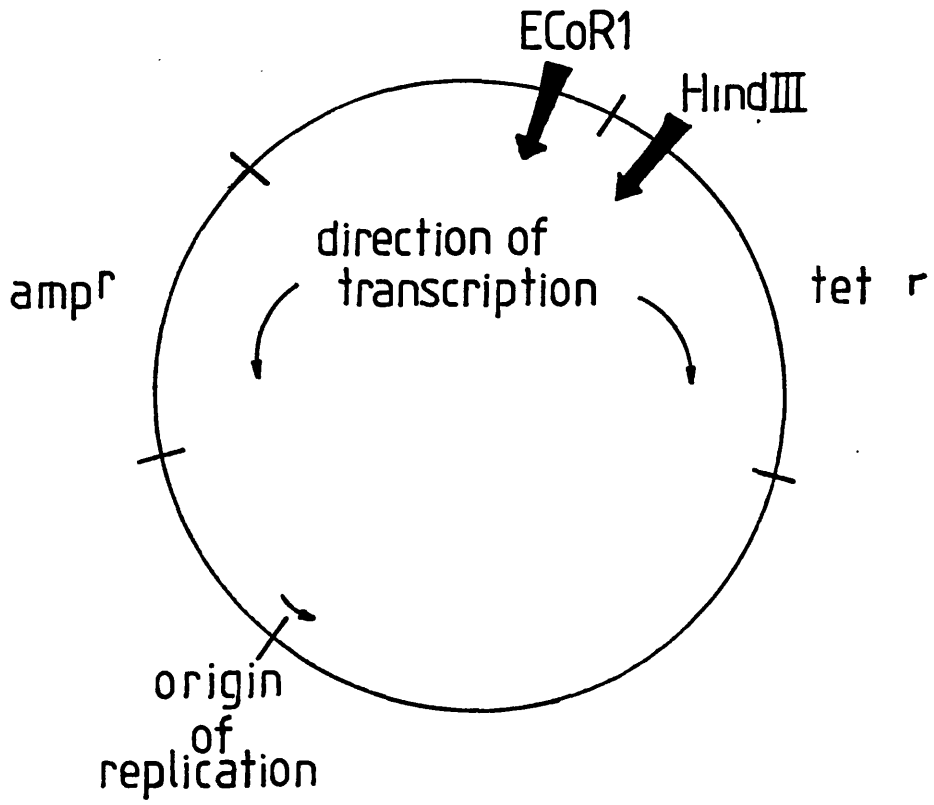
Cloning and restriction analysis of the wild type E.coli gene for CS revealed that the whole gene with its promoter site falls within a 3.2kb fragment, between an ECoRl restriction site and a HindIII site (Guest, 1981; Bloxham et al., 1984)(Fig. 8.1). Such a fragment is suitable for cloning in plasmid pBR322 (Fig. 8.2), recombinant plasmids being selected for by ampicillin resistance (amp^r) and tetracycline sensitivity (tet^s) of transformed cells.

Figure 8.1: Restriction Map of the E.coli Gene gltA



The gltA gene, coding for CS, is found within a 3.24kb fragment between restriction sites for HindIII and ECoRI. Plasmid pDB2 consists of this fragment cloned into pBR322 (see Figure 8.2).

Figure 8.2: Genetic Map of Plasmid pBR322



Insertion of DNA between the HindIII and ECoRI sites results in recombinant plasmids that are amp^r and tet^s.

As stated in 1.8.4, it is believed that the mutant CS was generated by relatively minor genetic alterations. If this is the case, then the restriction sites for ECoRl and HindIII may also be present in the mutant gene. This assumption was the basis of a strategy used in an attempt to clone the mutant gene.

8.1.3 The Cloning Strategy

1. DNA was purified from the mutant E.coli, Kllr3.

2. pBR322 was prepared by the method described in 3.25, after growth in E.coli HB101.

3. Both genomic Kllr3 DNA and pBR322 were digested with the restriction enzymes ECoRl and HindIII. Success of the digestion was determined by agarose gel electrophoresis (Fig 8.3).

N.B. The two restriction enymes have slightly different requirements for the buffer used in a digestion. The experiment described was carried out in 2 ways: firstly, with a simultaneous digest with both restriction enzymes; secondly, with a digest in which first one enzyme was used, the DNA was then removed by ethanol precipitation, resuspended in the appropriate buffer, and digested with the second enzyme. There were no notable differences between the results obtained from these two methods.

4. The 3.2kb region of the digest of Kllr3 DNA was cut out of the gel with a scalpel and the DNA separated from the agarose. It was mixed with the digest of the plasmid, and the mixture was ligated with T_4 DNA ligase.

5. The mixture was then used to transform a CS^- strain of

Figure 8.3: Agarose Gel Electrophoresis of HindIII and ECoRI digested K11R3 DNA

Lane 1

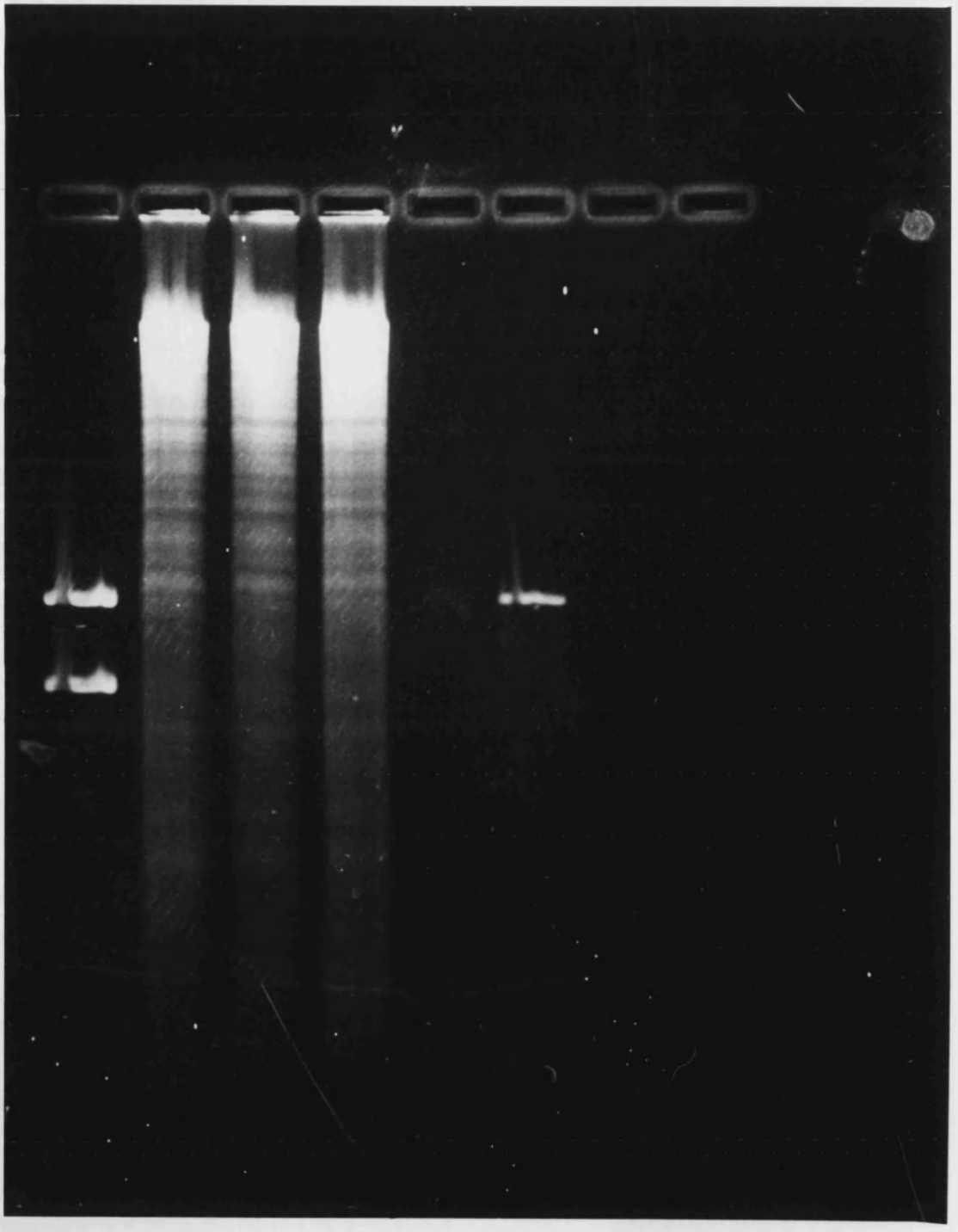
Digested pDB2 (the 3.2kb fragment, containing the gltA gene of wild-type E.coli, cloned into pBR322). The upper band is the pBR322 vector, and the lower band is the 3.2kb fragment.

Lanes 2 to 4

Digested K11r3 DNA.

Lane 6

A digest of pBR322; the DNA is all in the linear form.


1
2
3
4
6

E.coli (W620). As controls, no DNA, and the plasmid, pDB2 (the 3.2kb fragment of the wild type gene recombined in pBR322) were also used to transform the cells.

6. Transformants were selected on the basis of amp^r. No transformants were obtained when DNA was not added, and 4×10^4 transformants were obtained per μg of pDB2.

7. Any transformants with DNA inserted into the HindIII site of the plasmid would be tet^s, and this was used as a test for transformants with recombinant plasmid. (In fact the lesion in the tet^r gene caused by restriction with HindIII is in the promoter and not the structural region of the gene. Introduction of DNA with a promoter site into this lesion may therefore result in an active tet^r gene. This has been used to search for promoters in gene libraries. Nevertheless, introduction of the wild type CS gene into this region resulted in the loss of tetracycline resistance, and it is to be expected that the mutant gene will behave similarly.

8. Any amp^r, tet^s clones were tested for growth on glucose minimal medium without glutamate. Because of the absence of CS, W620 requires glutamate for growth. Transformants with the CS gene from Kllr3 present in a recombinant plasmid should be able to grow.

Results

From two separate experiments, 279 amp^r clones were obtained, 24 of which were tet^s. Seven of these 24 grew on glucose minimal medium in the absence of glutamate, and all seven contained CS; at least one of

them had a CS that was remarkably similar to that of Kl1r3. However, on curing these strains of plasmid by ethidium bromide treatment, no loss of the CS occurred. W620 is a rec^- strain (i.e., extrachromosomal DNA cannot recombine with the genome), and so it is unlikely that this was because of recombinational events. The only explanation is that W620, containing recombinant plasmid, had reverted, regaining active CS. W620 has a high frequency of reversion in glucose minimal medium (1 in 0.53×10^6).

8.1.4 Use of an Alkaline Phosphatase Step

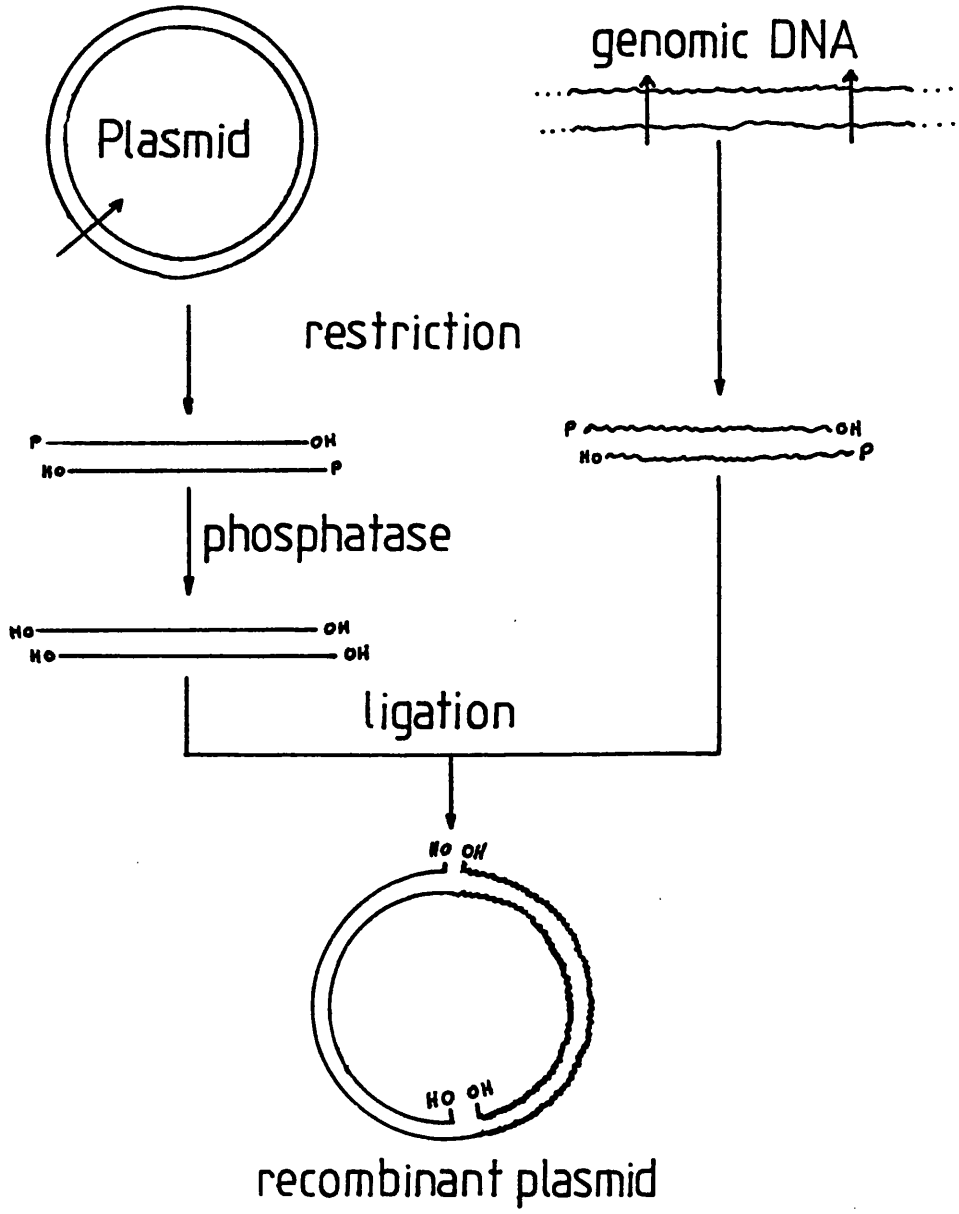
One problem with the cloning strategy outlined above is that on ligation, as well as recombination of plasmid with genomic DNA, plasmid is likely to recombine with plasmid, and genomic DNA with genomic DNA. In order to avoid this, and so increase the number of recombinant plasmids, the plasmid DNA once digested can be treated with alkaline phosphatase which will remove 5' PO_4s. Therefore upon ligation, only genomic DNA can be ligated to plasmid DNA (Fig. 8.4).

Alkaline phosphatase treatment was included in the cloning strategy, and in this case 200amp^r transformants were obtained of which 97 were tet^s; one of them grew faintly on glucose minimal medium in the absence of glutamate, but it had insignificant levels of CS.

8.1.5 Cloning with the whole Kl1r3 digest

The mutant gene may not be found in a 3.2kb

Figure 8.4: Use of Alkaline Phosphatase in the Cloning Procedure



fragment because of destruction of one or both of the restriction sites. The cloning experiment was therefore repeated using the whole of the K11r3 DNA digest rather than just the 3.2kb region. Of the 250 amp^r transformants obtained from this experiment, 150 were tet^s, but none of them grew on glucose minimal medium in the absence of glutamate.

8.1.6 Use of Single Digests

A further restriction site for HindIII or ECoR1 may have been introduced into the mutant gene, causing its destruction when that restriction enzyme is used. Cloning was therefore also attempted using a digest of K11r3 DNA by HindIII alone. In this experiment, approximately 50% of the amp^r transformants obtained were tet^s, but none of them grew on glucose minimal medium in the absence of glutamate. A digest with ECoR1 alone was also used; 1020 amp^r transformants were tested but found to be negative for growth in the absence of glutamate.

Similar experiments, but with selection by plating the transformation mixture directly onto glucose minimal medium, with ampicillin but without glutamate, also failed to provide any positive clones.

8.1.7 Shortcomings of the Cloning Strategy

The strategy itself suffers from 2 major drawbacks. In order to provide a simple selection procedure for the cloned gene, a CS^- strain was chosen for the transformation. This strain however transforms with a relatively low frequency (8×10^4 per µg pBR322, compared to 1×10^7 with E.coli HB101), increasing the chances of not detecting the desired recombinant plasmid. Purification of pBR322 after growth in W620 rather than HB101 did not improve the transformation efficiency.

It is also possible that the assumption on

which the strategy was based is wrong; we do not know how 'minor' the genetic changes were that produced the mutant; it is conceivable that one or both of the restriction sites have been destroyed, or that a further site(s) has been created within the gene. In order to test this further, the E.coli wild type gene was used as a probe in a hybridization experiment.

8.1.8 Preparation of the Probe for Hybridization

The 3.2kb fragment containing the wild type E.coli CS gene was prepared from plasmid pDB2 by digestion with ECoR1 and HindIII followed by separation of the fragments on an agarose gel. The 3.2kb band was cut out of the gel and the DNA recovered from the agarose. This fragment was then radiolabelled with ^{32}P dCTP by nick-translation, and used as a probe in the following hybridization experiments.

8.1.9 Hybridization to a digest of K11r3 DNA

ECoR1 and HindIII double digests of K11r3 DNA, K12 DNA, Bacillus subtilis 168 DNA, and pBR322 were run on an agarose gel. A digest of W620 was also prepared, but as can be seen from the gel, the digestion did not work (Fig. 8.5). The DNA fragments were blotted from the gel onto nitrocellulose, and the nitrocellulose was then incubated with the probe to allow hybridization. After washing away excess probe, the hybridization was visualized by autoradiography of the nitrocellulose. A photograph of the autoradiograph obtained is shown in Fig. 8.6. The probe hybridized to the undigested pDB2 and

Figure 8.5: Agarose Gel of HindIII and ECoRI Digests for Southern Hybridization

Lane 1

pDB2: the top band is singly digested plasmid; the middle band is the pBR322 remanent; the bottom band is the 3.2kb fragment.

Lane 3

W620 DNA: the digest has not worked, and only high relative molecular mass DNA is seen.

Lane 4

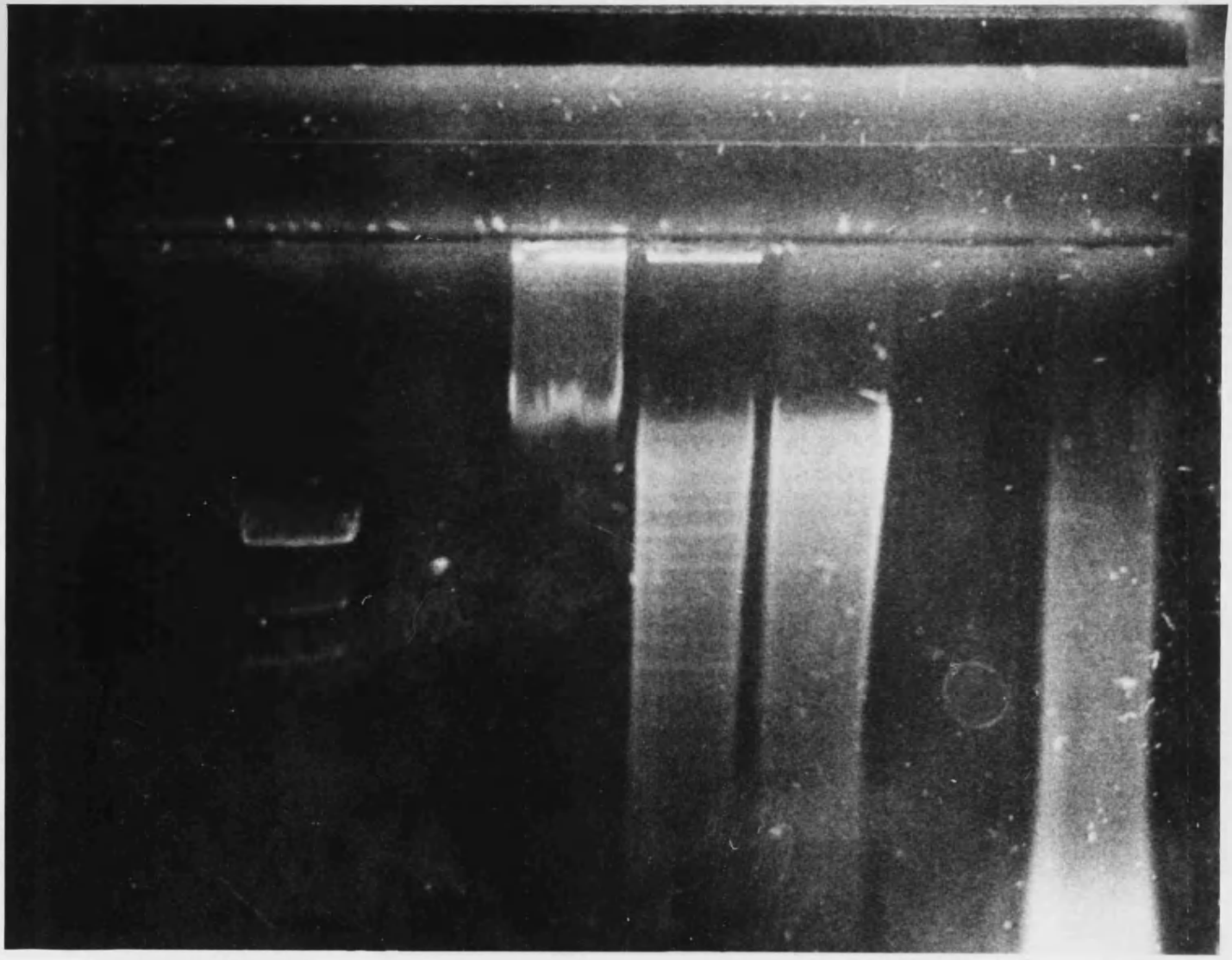
Digested K12 DNA

Lane 5

Digested K11r3 DNA

Lane 7

Digested B.subtilis 168 DNA. Lots of low relative molecular mass species appear to be present, perhaps indicative of a greater number of sites, for the restriction enzymes used, in the Bacillus DNA.



1 3 4 5 7

Figure 8.6: Autoradiograph Showing the Hybridization of the 3.2kb Fragment to a Nitrocellulose Blot of the Gel Shown in Figure 8.5

The top to the bottom of the gel is shown left to right (i.e., the same orientation as in Fig. 8.5). The bottom lane corresponds to the digest of pDB2; the hybridization is to the singly digested plasmid and to the 3.2kb fragment. The middle lane corresponds to the digest of Kl2 DNA. The upper lane corresponds to the digest of Kl1r3 DNA. In both of these cases hybridization is to an approximately 3.2kb fragment.

to the 3.2kb fragment, i.e., to itself; it hybridized to a 3.2kb fragment in the K12 digest and to a similar fragment in the K11r3 digest; it did not hybridize to any smaller fragments in either of these digests. This suggests that the 3.2kb fragment is present in K11r3, that neither of the restriction sites has been destroyed, and that no new sites have been created.

Nevertheless, it may be argued that the original mutation to produce a CS^- strain has altered this fragment, destroying the CS activity, but the reversion is due to another gene that has not been detected by hybridization with the 'original' gene. However, evidence from conjugation experiments (Danson et al., 1979a) indicate that the mutant gene maps in the same place as the wild type, and therefore it can be assumed that the fragment observed on hybridization does represent the mutant gene.

The undigested W620 DNA did not hybridize with the probe, or at least, there was no band on autoradiography. This is perhaps because of the lack of transfer of the high relative molecular mass species during the Southern blotting (large DNA fragments are known to transfer more slowly) rather than because of the absence of the gene, since colony hybridization experiments described in the next section show hybridization of the probe to W620.

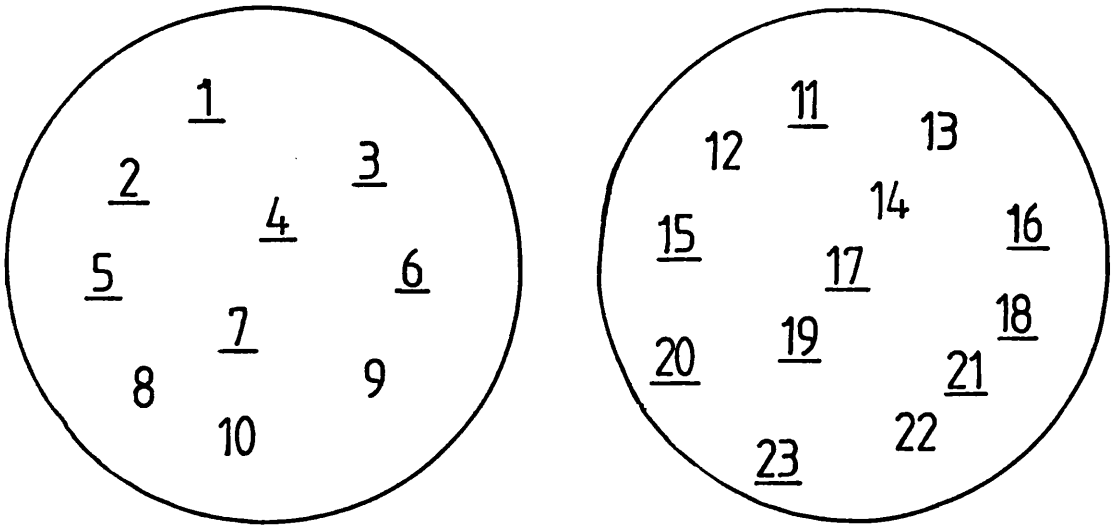
A high relative molecular mass species is seen faintly in the K11r3 lane in the autoradiograph, and it

is also visible in the K12 lane, although this does not show up on the picture. This is probably caused by hybridization to undigested or only partially digested DNA. No hybridization was seen with the digest of Bacillus subtilis 168.

8.1.10 Colony Hybridization (Fig.8.7)

In a colony hybridization study, the probe was shown to hybridize to E.coli strains K12, DB1002, W620, W620/pBR322, K11r3, HB101, and HB101/pDB2. No hybridization was detected with Bacillus subtilis 168, Bacillus subtilis Cu1695, or with Acinetobacter calcoaceticus 4B. The result with Bacillus subtilis is consistent with that from the Bacillus subtilis 168 DNA digest described in the previous section. The archaebacteria, Thermoplasma acidophilum, Sulpholobus acidocaldarius, and Natranococcus occultus, were also tested, but no hybridization was detected. However, the method used may not be applicable to the archaebacteria: the cells had to be spread onto the filters, rather than grown on them, because of the extreme conditions required for archaebacterial growth, and also the archaebacterial membrane and, when present, the cell wall differ from those of the eubacteria. A survey of 13 pseudomonad species with large CS, small CS, or both, revealed hybridization in all cases where a large CS was present, but not where only a small CS was present (Fig. 8.7). The one exception to this was Ps. maltophilia, which though believed to contain only a small CS, gave a detectable

Figure 8.7: Colony Hybridization



1. E.coli K12
2. E.coli W620
3. E.coli DB1002
4. E.coli W620/pBR322
5. E.coli HB101/pDB2
6. E.coli K11r3
7. E.coli HB101
8. B.subtilis CU1695
9. B.subtilis 168
10. Acinetobacter calcoaceticus 4B

11. Ps.Aeruginosa 1978 (L&S)
12. Ps.iodinum (S)
13. Ps.chlororaphis D302 (S)
14. Ps.saccharophila D1021 (S)
15. Ps.acidovorans D1870 (L)
16. Ps.maltophilia D144 (S)
17. Ps.aeruginosa PAC514 (L&S)
18. Ps.stutzeri (L&S)
19. Ps.diminuta D1032 (L)
20. Ps.putida (L&S)
21. Ps.aeruginosa PAC1 (L&S)
22. Ps.alcaligenes D123 (S)
23. Ps.testosteroni D1047 (L)

L indicates presence of a "large-type" CS.
S indicates the presence of a "small-type" CS.

spot. Despite this anomaly, it seems likely that hybridization occurred at one locus coding for the large type, whilst there is a second locus for the small type, to which there was no hybridization. The two loci may not both be present on the chromosome, but perhaps one is on the chromosome and the other on a plasmid.

These studies show that the 3.2kb fragment is not a good general probe for CS genes. Sequencing studies have revealed considerable homology between the CSs of yeast and pig heart; homology to the E.coli enzyme is less marked, but still significant. By study of he available sequences, it may be possible to decide on suitable sequences for use as more general probes of CS genes.

8.1.11 The Mutant Enzyme is Almost Entirely Suppressed by Glucose

The activity of the mutant enzyme was usually measured in extracts of cells that had been grown in L-broth or nutrient broth. These media are devoid of carbohydrate, and so the citric acid cycle is required for energy production. However, selection for the gene was carried out in glucose minimal medium, which is known to cause a suppression in the levels of wild type CS.

Extracts of K12, K11r3, W620, and DB1002, all of which had been grown in glucose minimal medium, were assayed for CS (Table 8.1). Under these conditions, the level of CS in the mutant strain was virtually zero, and was less than that in the supposedly CS^- strain, W620.

Table 8.1: Specific Activities of Citrate Synthase in Crude Cell Extracts

Growth Medium	CS (mU/mg protein)				
	E.coli				B.subtilis
	K12	DB1002	K11r3	W620	168
L-broth	388	7,790	13.5	<1	109
Glucose minimal medium	80	425	<1	<1	20.4
Succinate minimal medium	867	2,840	24	11	201

Assays were carried out as described in 3.4.1. KET8 buffer was used for the E.coli strains, and ET8 buffer was used for B.subtilis 168.

Some CS must be present in this strain in this media, since (unlike W620) there was growth in the absence of glutamate. The growth is however relatively slow, and only poor yields of cells are obtained. Considering this, it is not suprising that the gene was not selected by means of complementation in glucose medium.

8.1.12 Escherichia coli, W620 Expressed Significant Levels of Citrate Synthase When Grown in Succinate Minimal Medium

An obvious answer to the problem of suppresion in glucose minimal medium is to use a different carbon/energy source, succinate for example. However, a survey of the activity of the CS activity of extracts of K12, K11r3, W620, and DB1002, all grown in succinate minimal medium, revealed that, although K11r3 had substantial CS activity, so did W620 (Table 8.1).

The high frequency of reversion of W620 has already been referred to (8.1.3). It appears that the apparently CS^- strain that was being used was in fact itself a peculiar type of revertant, exhibiting CS activity when grown in succinate minimal medium, but not when grown in glucose minimal medium, nutrient broth, or L-broth.

8.2 Studies with Bacillus subtilis

The importance of B.subtilis CS as the 'link' between the CSs of E.coli and pig heart has been indicated (1.9). Comparisons at the level of the gene as well as the level of the functional protein are clearly

desirable.

8.2.1 Attempts to Clone the Citrate Synthase Gene from Bacillus subtilis 168

A similar cloning strategy to that outlined above was used to attempt to clone the CS gene from Bacillus subtilis 168. In this case, we have no reason to suppose that the gene is bordered by ECoR1 and HindIII restriction sites, or that it has no such sites within the gene itself. Therefore, three different digests of Bacillus subtilis DNA were used: a HindIII digest, an ECoR1 digest, and a digest with both of these restriction enzymes. The sucess of the digestion was tested by applying a sample to an agarose gel. The rest of the digest was then mixed with alkaline phosphatase-treated pBR322 (which had been digested with the same restriction enzyme(s) as the digest with which it was mixed), and the mixture was ligated with T_4 DNA ligase. The ligation mixture was used to transform E.coli W620, and selection was carried out as before (8.1.3 ~~8.1.6~~). At the time that this experiment was performed, the information described in 8.1.12 was not known. However, it is unlikely to have had any effect here, since glucose minimal medium could be used with the confidence that Bacillus subtilis CS, though suppressed by glucose, should still be present at significant levels (see Table 8.1). 111 amp^r, tet^s clones were obtained from the HindIII digest, ~~6 from the ECoR1 digest~~, and 8 from the double digest; none of these grew on glucose minimal medium in the absence of glutamate.

No glutamate prototrophs were detected in the transformants obtained from the ECoR1 digest.

A major problem here is that even if the Bacillus subtilis CS gene is cloned intact into the E.coli W620, it may not be expressed. However, experience has shown that it is more likely for a Bacillus gene to be expressed in E.coli than vice versa. Even if the gene is expressed and the CS protein produced in a properly processed form, being of a different type to the native enzyme, growth may still be poor or not possible. Therefore in order to clone the gene from Bacillus subtilis, it would be more sound to first select for the gene in a Bacillus strain, even if it is later to be manipulated in an E.coli strain. To make this possible a Bacillus subtilis CS^- strain is desirable in order to be able to determine the presence or absence of the gene.

8.2.2 The Bacillus subtilis 168 mutant, CU1695, is deficient in Citrate Synthase

Work by Carls & Hanson (1971) suggested that, in Bacillus subtilis, the gene for CS was linked to that of aconitase. CU1695 is a strain derived from Bacillus subtilis 168, known to have a large deletion in the area of the aconitase gene (Fig. 8.8). Extracts of CU1695 were tested for CS and other citric acid cycle and related enzymes, and the levels were compared with those of 168 (Table 8.2). CS was not detected in the extract from CU1695, nor were aconitase, 2-oxoglutarate dehydrogenase (2OGDH), and glutamine oxoglutarate aminotransferase (GOGAT), the genes of which all lie in the deleted region. Isocitrate dehydrogenase (IDH), malate

Figure 8.8: Partial Genetic Map of the Bacillus subtilis Chromosome

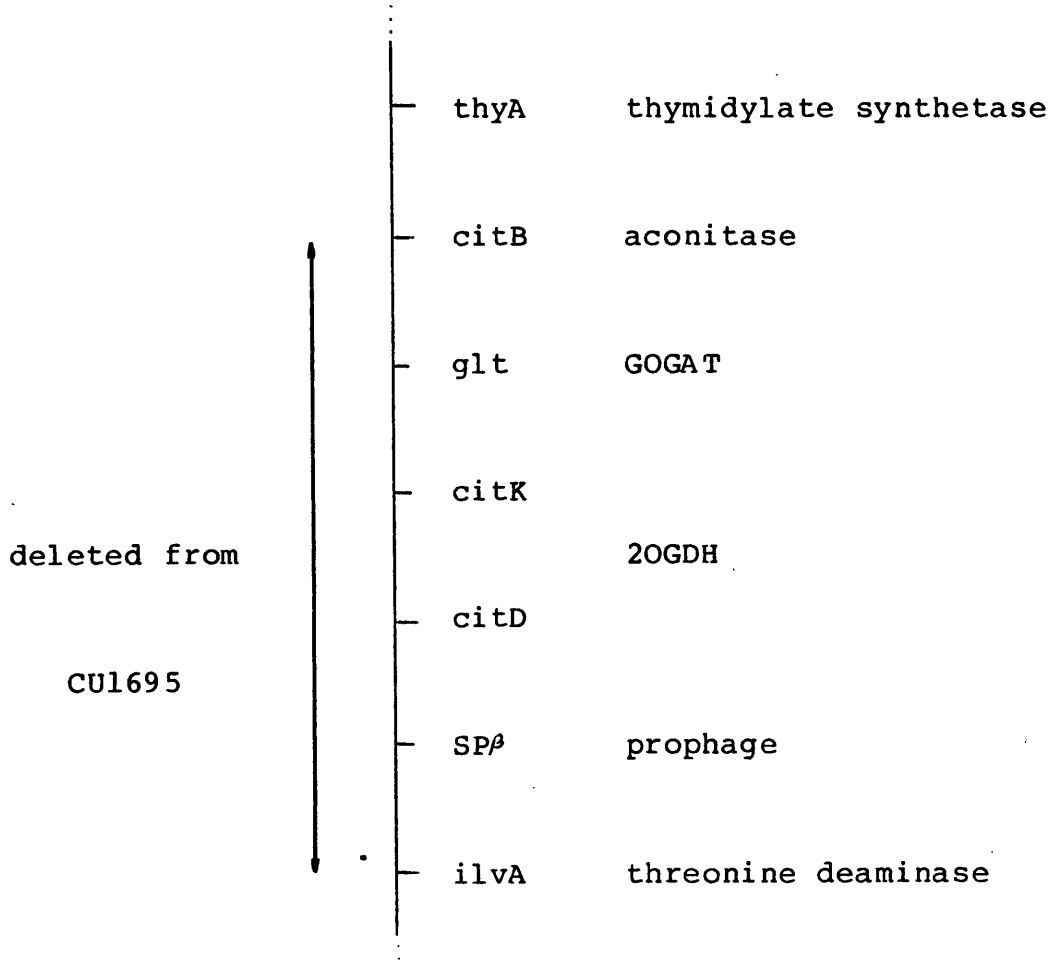


Table 8.2: Comparison of Enzyme Activities in Crude Extracts of Bacillus subtilis Strains 168 and CU1695

Enzyme	Specific activity (U/mg)	
	168	CU1695
aconitase	0.05	0
CS	0.167	0
deacylase	0.004	0.012
GOGAT(NADPH)	0.005	0
IDH	14.4	3.4
LDH	0.25	0.20
E3	0.25	0.91
MDH	3.73	0.90
2OGDH	2.4	0

Crude extracts of 168 or CU1695, prepared from 18h-cultures in L-broth, were assayed for enzyme activities as described in 3.4.

dehydrogenase (MDH), and lactate dehydrogenase (LDH) were all present in CU1695, but at slightly lower levels than in 168. Lipoamide dehydrogenase and deacylase were in fact higher in CU1695 than in 168. The result for deacylase is interesting in view of the hypothesis that deacylase is increased when there is a deficiency of CS (Robinson, personal communication). Lipoamide dehydrogenase is a component of both the 2-oxoglutarate dehydrogenase complex and the pyruvate dehydrogenase complex; its gene is located outside the lesion in the genome of CU1695.

In order to test whether the absence of CS from CU1695 is caused by a regulatory effect related to the absence of 2OGDH and/or GOGAT, Bacillus subtilis strains CU1323 (2-OGDH$^-$, GOGAT$^-$) and UTB600 (GOGAT$^-$) were also tested for CS. Both were found to have CS at levels comparable with those of 168: 0.029 U/mg (1/6th 168) with CU1323; 0.083 U/mg (1/2 168) with UTB600. The decrease in the levels is probably not significant, but may be caused by a general decrease in biological fitness of the strains compared to 168; in any case, it is nothing like as dramatic as the complete absence of CS from CU1695. It seems likely therefore that CU1695 has at least a partial deletion of the CS gene, resulting in the absence of detectable active protein. The CS gene must therefore map between citB and ilvA (Fig. 8.8).

8.2.3 Transformation of CU1695 with 168 DNA

The large lesion of the CU1695 genome forces

numerous growth requirements upon it, and these render it useless for the purpose of screening for the CS gene. If part of this lesion were filled, e.g., by transformation with 168 DNA, then the strain may become of some use in this context. Moreover, if by transformation CS activity were regained, this would be further evidence that the CS gene was indeed in the area of the lesion.

CU1695 was transformed with 168 DNA, and plated onto agar with minimal medium and all the relevant growth requirements except one, and also onto control plates that had all the growth requirements. The number of viable cells of the transformed culture was also determined by plating onto nutrient agar. No colonies grew on any of the test plates even after several days of incubation. However, the number of colonies on the control plates was only approximately one thousandth of the number of viable cells plated; the poor efficiency of plating may explain the lack of detectable transformants. Of course, transforming with large chromosomal DNA rather than with closed circular plasmids is an inefficient process to begin with.

8.2.4 Attempts to Isolate A Citrate Synthase$^-$ mutant of Bacillus subtilis

8.2.4.1 The Effect of Monofluoroacetate on Bacillus subtilis 168

Fluoroacetate has been used in the isolation of CS$^-$ mutants of Acinetobacter calcoaceticus 4B (Harford & Weitzman, 1980). The method depends on the extreme

toxicity of fluorocitrate generated from the fluoroacetate via CS. If CS is absent, then the toxic fluorocitrate cannot be formed. However, it was found that 168 would grow well in the presence of at least 10mM fluoroacetate (10x the amount used by Harford & Weitzman, 1980). There must already exist some block in the uptake or metabolism of fluoroacetate such that fluorocitrate is not synthesized. Alternatively, fluorocitrate may not be toxic to Bacillus subtilis. The mechanism of action of fluorocitrate is not entirely clear; even though it is a powerful inhibitor of aconitase, other effects such as inhibition of citrate transport may also be important.

This method cannot therefore be used directly to obtain mutants of Bacillus subtilis. It may however, be worthwhile to generate fluoroacetate-sensitive mutants of Bacillus subtilis which could then be used to obtain CS^- mutants.

8.2.4.2 Mutation with Ethyl Methanesulphonate

Mutagenesis with EMS followed by a penicillin (methicillin) enrichment procedure for glutamate auxotrophs resulted in the selection of 2 such strains. Both had normal levels of CS; one was deficient in GOGAT.

8.2.4.3 Mutation by UV Irradiation

UV irradiation, to give a 1% survival rate, followed by penicillin enrichment for glutamate auxotrophs was also unsuccessful in obtaining a CS^- mutant. One glutamate auxotroph was isolated by this procedure, but it was found to be deficient in GOGAT and

not in CS.

8.2.4.3 Possible Reasons for the Failure to Isolate a Citrate Synthase$^-$ Mutant of Bacillus subtilis

The reasons for the failure to isolate a CS$^-$ mutant are unknown. It may be that methods involving glutamate auxotrophy are not applicable to Bacillus subtilis, but there is no known reason why this should be so. Relatively few glutamate auxotrophs in total were isolated and so the methods used need to be extensively optimized to this end. Unfortunately, the most powerful and specific method (i.e., using fluoroacetate) could not be used.

8.3 Future Work

Despite the lack of success in the experiments described in this chapter, valuable information has been obtained, and the studies can be used as a basis for further work. If the complementation method of selection is to be used, then the physiology of the CS$^-$ strains and the mutant strains needs to be more extensively investigated. A more stable CS$^-$ mutant of E.coli is required. As suggested in 8.1.8, the considerable homology observed between the CS genes that have been sequenced so far may enable the construction of specific and general probes to aid in the cloning of CS genes. Methods involving antibodies to CS may also be used to select for the presence of the desired gene..

As far as cloning of Bacillus subtilis CS is concerned, if CS$^-$ mutant cannot be obtained, then a more

sophisticated vector than pBR322 may be used to achieve expression of Bacillus subtilis CS in E.coli.

The cloning strategy used in this study is only one of the possible strategies, though it is the simplest. Another approach would be to obtain a random gene library, e.g., by sonication of the DNA, which may then be screened for the CS gene.

9. PRODUCTION OF A COMPOUND METABOLICALLY RELATED TO GLUTAMATE BY CULTURES OF ESCHERICHIA COLI

9.1 Introduction

The experiments described in this section were designed to confirm and follow up the studies of Harford (1977) that showed that a compound was excreted from colonies of the mutant of E.coli with the small type CS. This compound supported the growth of a CS^- (and therefore glutamate requiring) strain of E.coli which had previously been lawned onto the same agar plate. The medium used for the agar plates did not contain glutamate (or a compound that could be metabolized to glutamate without the interpolation of CS), and so would not support the growth of the CS^- strain. A ring of satellite colonies appeared around the colonies of the mutant E.coli strain, but not around the wild type colonies.

9.2 Pre-preparation of the Bacterial Strains Used

The strains used in these experiments were adapted for growth on glucose minimal medium by subculturing them twice onto glucose minimal agar supplemented with the appropriate growth requirements.

9.3 Production in Agar

Cells from 10ml of a stationary phase culture of W620 (a CS^- mutant of E.coli) in glucose minimal medium were collected by centrifugation, washed in 0.85%(w/v) sterile saline, and plated onto glucose minimal agar plates that were supplemented with the

appropriate growth requirements for the strain to be added (see below) and with uracil and thiamin, but not with glutamate, thus preventing the growth of W620. After incubating the plates overnight, samples of the appropriate bacterial strains were added to them with a sterile loop and spread out over a small area of the plate (about $0.5cm^2$). The plates were then returned to the incubator for a further 36h. After this period of incubation, an area of growth of tiny colonies had appeared around the smears of the E.coli strains that have a wild type CS, but not around the smears of the E.coli strain K11r3 which has a small CS or around the smears of the other bacteria used (Table 9.1). Several larger colonies had also appeared in random positions on the plates, presumably due to reversion of the W620 to CS^+. The frequency of reversion was high (1 in 0.53×10^6).

Some of the satellite colonies produced were transferred to nutrient agar and grown overnight. Cells were then taken from these plates to test the growth requirements of this organism by plating onto glucose minimal agar plates ±glutamate, and ± uracil and thiamin. The organism was found to have the same phenotype as W620, i.e., a requirement for glutamate, uracil, and thiamin.

These experiments show that, under these conditions, E.coli strains with wild type CS overproduce a compound which is exported into the medium. This

Table 9.1: Production of Satellite colonies

Organism	Special Growth Requirements	Satellite Colonies Produced
E.coli		
K12	none	yes
DB1002	uracil & thiamin	yes
K11r3	met & thy	no
B.subtilis		
168	trp	no
Acinetobacter calcoaceticus 4B	none	no
Pseudomonas maltophilia	none	no
Pseudomonas testosteroni	none	no

The experiments were carried out as described in 9.3.

compound must either be glutamate itself, or be convertible into glutamate in the absence of CS, since it can support the growth of the CS^- strain, W620.

9.4 Production in Broth

E.coli K12, DB1002, and K11r3 were grown in 10ml of glucose minimal medium supplemented with the appropriate growth requirements. After the cells had reached stationary phase, they were removed from the 'used' medium by centrifugation followed by filtration of the supernatant through a 0.22μm filter. The used media was then concentrated approximately 10x by rotary evaporation, and was used for the experiments described below.

W620 was inoculated into 5 flasks: one with 5ml glucose minimal medium + glutamate, uracil, and thiamin; one with 5ml glucose minimal medium + uracil and thiamin; three with glucose minimal medium + uracil and thiamin, to which 0.5ml of the concentrated used medium from either K12, DB1002, or K11r3 had been added. After incubation for 36h, growth was observed in the flask with glutamate added, and in the flasks with media from K12 and DB1002, but not in the flask without glutamate, or the flask with medium from K11r3.

9.5 Identification of the Compound Produced

On paper chromatography, using 100μl of the concentrated medium from 9.4, a faint spot, which had the same Rf as 2-oxoglutarate, was observed with the media from K12 and DB1002, but not with the media from K11r3 or

with the unused medium control (see Table 9.2).

Thin layer chromatography of DNPH derivatives of 100µl of the concentrated media from K12 and DB1002, produced a spot which ran marginally ahead of the 2-oxoglutarate standard, but could not be separated from it when the sample was mixed with the standard. The K11r3 medium gave a faint spot in the same place. (Table 9.3) However, no 2-oxoglutarate could be detected in the used media by an enzymic method using glutamate dehydrogenase.

9.6 Comparison with Previous Results

These results are in total disagreement with those of Harford (1977) who observed the satellite growth phenomena using succinate minimal medium in the case of a mutant E.coli with a small CS, but not with wild type E.coli. In that experiment, he used a different CS^- strain, and a different isolate of the mutant E.coli. The mutant CS from that isolate was however indistinguishable from that of the strain used in this study as far as its kinetic and regulatory properties, and DTNB inhibition are concerned.

It was hoped to repeat the experiments described here, but with succinate minimal medium, as in the experiments of Harford (1977), rather than glucose minimal medium. But it was found that W620 has a significant activity of CS when grown in this medium (Table 8.1).

Table 9.2: Paper Chromatography of Organic Acids

Acid/Sample	Rf
aconitic	0.46
citric	0.34
oxaloacetic	0.67
2-oxoglutaric	0.63
glutaric	0.11
succinic	0.70
Kl2 medium	0.62
DBl002 medium	0.63
Kllr3 medium	no spot observed
Unused medium	no spot observed

Chromatography was carried out by the method of Buch et al. (1952) as described in 3.21. Samples were prepared as described in 9.4.

Table 9.3: Thin-layer Chromatography of 2,4-Dinitrophenylhydrazine Derivatives

Acid/Sample	Rf
oxaloacetic	0.71
2-oxoglutaric	0.76
pyruvic	0.84
Kl2 medium	0.78
DBl002 medium	0.78
Kllr3 medium	0.78
Unused medium	no spot observed
DBl002 medium + 2-oxoglutaric	0.77

Chromatography was carried out as described in 3.22.
Samples were prepared as described in 9.4.

9.7 2-Oxoglutarate Inhibition of E.coli Citrate Synthase is Unaffected by the Type of Growth Medium Used

The mutant CS is desensitized to 2-oxoglutarate inhibition as compared to the wild type, and this observation was used to rationalize the findings of Harford (1977). 2-Oxoglutarate inhibition of both mutant and wild type CS was investigated in extracts of cells grown in L-broth, glucose minimal medium, and succinate minimal medium. The results show that the inhibition is not noticeably affected by the growth medium used (Table 9.4).

9.8 Discussion

A compound that is related to glutamate, probably 2-oxoglutarate, is overproduced by E.coli strains with wild type CS, but not by a mutant strain, Kllr3, nor by the other bacteria tested. It would appear that whatever is occurring pertains to some facet of the control of the citric acid cycle of E.coli that does not exist in the other bacteria. The main thing that stands out in this context is the split cycle mode. This is discussed below.

It can be seen from Table 8.1 that glucose causes a drastic decrease in the amount of CS present, reducing the levels in Kllr3 to almost zero (see also Section 8.1.11). It is therefore not suprising that there is no observable overproduction in Kllr3; the CS is probably insufficient to generate an excess of the compound. The Kllr3 strain is also slower growing than

Table 9.4: Inhibition of Escherichia coli Citrate Synthase by 2-Oxoglutarate

Growth Medium	[2OG]/mM	% Inhibition		
		K12	DB1002	K11r3
L-broth	0	0	0	0
	0.5	42	40	0
	1.0	73	63	2
	2.0	84	84	8
Glucose minimal medium	0	0	0	-
	0.5	44	59	-
	1.0	55	63	-
	2.0	78	83	-
Succinate minimal medium	0	0	0	0
	0.5	37	23	0
	1.0	61	60	2
	2.0	73	78	18

Assays were carried out as described in 3.4.1 using ET8 buffer. K11r3 has negligible CS when grown in glucose minimal medium.

the wild type, and so it may not be able to turnover enough substrate to generate the excess. In the case of the wild type E.coli, the decrease in levels due to glucose, and the inhibition by increased 2-oxoglutarate are clearly insufficient to prevent the overproduction. The inhibition by 2-oxoglutarate is perhaps of less significance in vivo where the citric acid cycle enzymes could be present in a multi-enzyme complex (Robinson & Srere, 1985; S.J. Barnes, personal communication). Indeed, it has been shown that the inhibition is less in a high relative molecular mass complex isolated from E.coli than with soluble E.coli CS (S.J. Barnes personal communication).

Perhaps of more significance is the shutting down of 2-oxoglutarate dehydrogenase in E.coli grown in anaerobic conditions, and the split cycle mode (Fig. 1.2) which may also operates to some extent in aerobic conditions. Possibly the growth of the cells on agar and in the culture flasks was substantially anaerobic, especially during the later phases of growth.

To establish more clearly what is occurring, a variety of CS^- strains of E.coli, and of E.coli mutants with altered CSs needs to be used, and the chemostat used to grow cells in known conditions of oxygen tension and substrate concentration.

10. GENERAL DISCUSSION

"Not chaos-like, together crushed and bruised,
But, as the world harmoniously confused:
Where order in variety we see,
And where, though all things differ, all agree."

Alexander Pope (Windsor Forest)

10.1 The Diversity of Approach

As discussed in 4.1, to obtain the fullest possible understanding of any system, a variety of methods needs to be employed. In the study of enzymes, protein chemistry, kinetics, molecular biology, immunology, etc., all have their place, and the information they provide is complementary, not exclusive.

Each of the chapters, 5, and 7 to 9, describe different approaches to the study of CS. Each of them only touches the surface of their topics, asking more questions than they answer, but a common aim connects them; they are not just studies of CS *per se*, but are aimed at understanding the structure-function relationships exhibited by the different CSs. The long term aim is to explain, firstly, how the different functions arise, i.e., by what molecular mechanisms, and secondly, why they did so.

10.2 Interactions

"No man is an Island, entire of itself;
every man is a piece of the continent,
a part of the main."

John Donne (Devotions)

CS is, by definition, the enzyme that catalyzes the condensation of OA and AcCoA to form citrate and CoA. By its very nature, in any species, in any shape or form, it is obliged to perform this function. But the enzyme does not exist in isolation; it is part of a highly organized system. From organism to organism the system differs and so different constraints are placed upon its components. Only when the level of the system is considered does the concept of regulation arise; the catalytic action of an enzyme is a function of a single entity, the enzyme itself, whereas regulation is fundamentally concerned with the interactions of a system.

Strictly, it is not correct to describe an enzyme catalyzing a reaction as a single entity: the reaction involves the interaction of enzyme, substrates, products, and solvent(s). As soon as any multiplicity is introduced, we have constituted a system, all parts of which interact with all other parts. Even within a single molecule, the individual atoms of which it is composed constitute an interacting system. The pealing off of layers can be continued down to the depths of the atom, but two key points have already been revealed. Firstly,

that matter consists of a web of interactions, and secondly, that these interactions can be considered at different levels of structure.

When we speak of an organized system, higher levels of structure than the atom or molecule are usually envisaged. Such organization is achieved at the expense of order in the environment of the system, and requires increasingly subtle forms of control to guard against, at least temporarily, the onward march of entropy.

A study of the highly organized systems that constitute the living organism must therefore involve several layers of structure: the enzyme (CS), the metabolic pathway (the citric acid cycle), the organelle, the cell, etc.. When we look at the properties of CS, we look at the level of the enzyme, and a large amount of information is obtained at this level. But as we have said, for an understanding of regulation, we must move up at least one level in the hierarchy, and consider the enzyme as a component of a system. By such an approach, for example, the inhibition of E.coli CS by 2-oxoglutarate, and cyanobacterial CS by 2-oxoglutarate and succinyl-CoA can be rationalized in terms of modified citric acid cycles.

10.3 Enzyme Structure and Function are two Different Levels of Organization

From knowledge of the structure of an enzyme alone its function in the cell could not be predicted, but from knowledge of the function, certain aspects of

its structure may be determined, and moreover, any structural knowledge may then be usefully applied to the known functions. Generally, properties of a particular level can be related back to a lower level, but the reverse does not hold. For each level, a set of 'rules' exists which cannot be predicted from the rules of lower levels.

An analysis of structure-function relationships looks at a higher level than studies of structure alone. For example, a protein may be digested with some protease, and the pattern of fragments obtained may be recorded and compared to similar digests of other proteins. One might then say perhaps that this protein has a fragment of relative molecular mass, 16,000, but the other proteins do not. Such results are meaningless to anyone who is interested in the function of proteins (though of course, they may be of great interest to a chemist or protein chemist who is analyzing at levels lower than the macromolecule itself). But by relating the results obtained to higher level information (the function of proteins), the proteolytic patterns spring to life and provide us with a completely different kind of information.

10.4 The Family of Citrate Synthases

In 1.2.2, the diversity of CSs was introduced in terms of a family of similar, but distinct enzymes. In a sense, this is a formal analogy, since it seems likely that the different enzymes all diverged from the same

'ancestor' molecule.

The studies described here, and also previous studies, have been designed to probe the differences in function of the different members of the family and relate them to differences in structure. The mutant E.coli enzymes were considered to have a key role in these studies, since functionally insignificant differences in structure should be minimized.

The studies so far have demonstrated that in spite of the diversity there is an underlying similarity between the members of the family. The existence of the small mutant enzyme and the behaviour in the trypsinolysis experiments suggest that the hexameric E.coli CS is really a trimer of dimeric CS units. The hexameric structure provides an extra level of organization which may allow (require) more sophisticated control mechanisms (NADH allosteric inhibition). The greater sensitivity of this enzyme to palCoA and related compounds may also be related to this extra degree of organization.

10.5 Exploring the 'Great Divide'

Despite the underlying similarities, the diversity exhibited by CS with respect to nucleotide inhibition (or palCoA inhibition) has not been explained. Inhibition by either or both of these nucleotides, ATP and NADH, would be an efficient control mechanism, but at the level of the enzyme system (the citric acid cycle) there is no known reason for the difference in behaviour.

That succinate thiokinase and possibly pyruvate dehydrogenase complex also follow the same pattern of diversity is suggestive of a fundamental difference between the Gram-positive bacteria and eukaryotes on one side, and the Gram-negative bacteria on the other. This problem must be investigated at higher levels of organization than the enzymes alone; perhaps at the level of the cell and its interactions with its natural environment, or perhaps the answer lies with the putative multi-enzyme complexes of citric acid cycle enzymes.

10.6 Some Other Unanswered Questions

Although it is one of the most well studied enzymes, the scope for further work on CS is still large. As well as the unexplained diversity in nucleotide inhibition, there are numerous unanswered questions; a few of these, that are relevant to the work described here, are highlighted below.

The role of palCoA is still obscure and may be of no physiological significance. Nevertheless, in that it interacts stongly with CS, it may prove to be an important analytical tool.

The experiments described in chapter 9 have called into question the *in vivo* significance of 2-oxoglutarate inhibition of *E.coli* CS, and as suggested there, its apparent lack of efficacy *in vivo* may be because the enzyme exists in the cell as part of a multi-enzyme complex.

From palCoA, to 2-oxoglutarate, to ATP, the

various experiments have demonstrated how difficult it is to decide whether or not an effect seen *in vitro* has any metabolic significance.

The molecular mechanisms of the allosteric changes of *E.coli* CS induced by NADH are still to be elucidated. In this context, further studies with the mutant enzymes may be particularly revealing. Also, if as the trypsinolysis studies suggest, there is a high degree of interaction between the subunits of the dimeric CS moiety, then the molecular mechanisms of this need to be investigated.

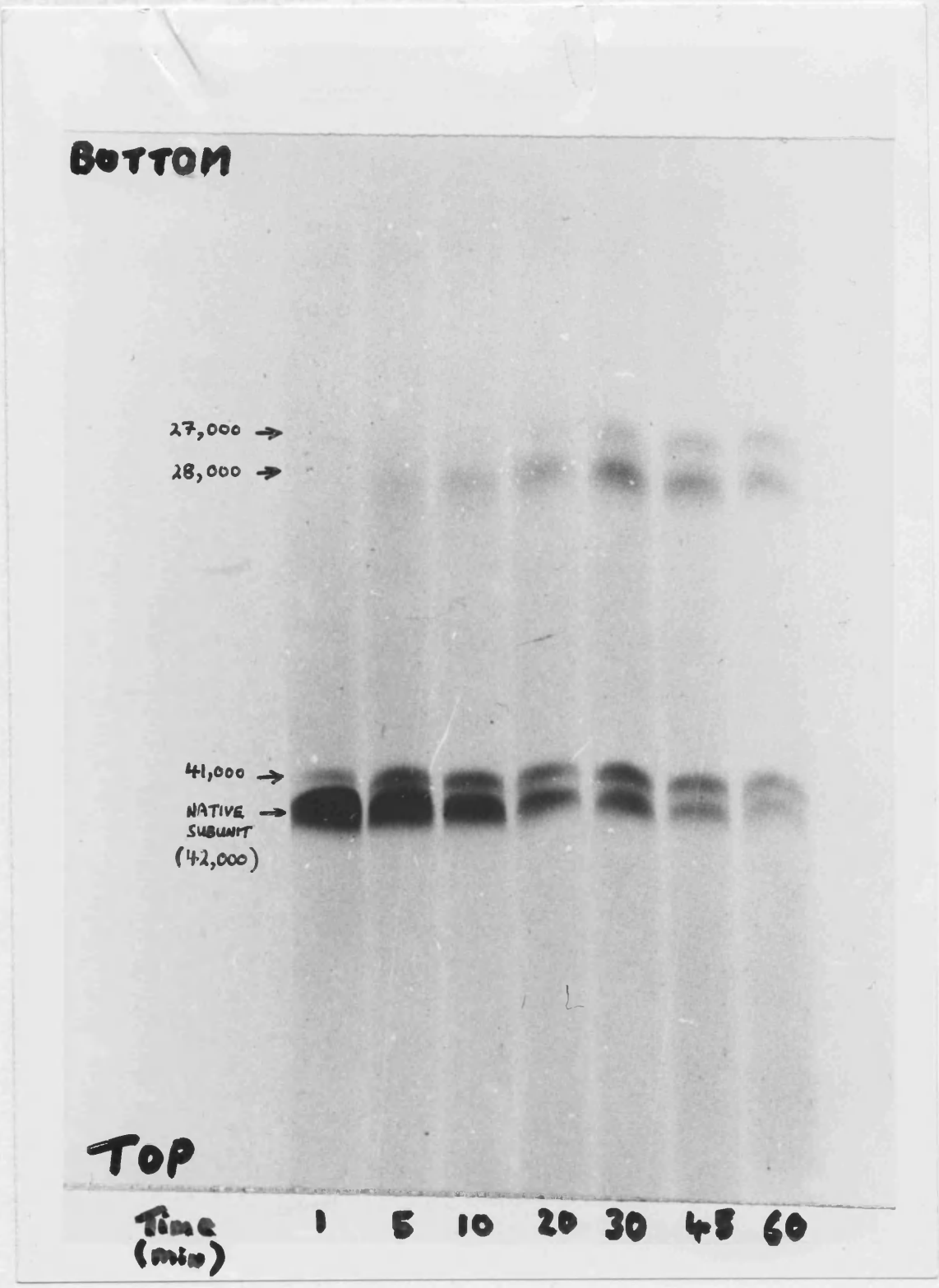
10.7 The World Harmoniously Confused

The world is not barren and uniform, but filled with a chaotic variety of structures. The living world is especially rich in its diversity, its extra layers of organization, over those of inanimate systems, producing a myriad of further possibilities. The diversity exhibited by CS is only one small manifestation of this almost infinite variety. Nevertheless, despite the diversity of CSs, we have seen that there is an underlying basic structure. Equally, the diversity of nature does not negate the essential unity of life; a molecular logic of living organisms is universally applied. This logic is the set of rules that governs the levels of organization that turn the molecules of life into the living cell, the set of rules that the biochemist wishes to discover.

One of the principles of this logic is

particularly apt in a discussion of CS. It has been said that CS is an important regulatory enzyme, and yet Kacser & Burns (1979) have rightly pointed out that all parts of a system are important, and they have referred to a molecular democracy of the cell. But as in human democracies, power still resides in the hands (binding sites) of a privileged minority. The principle to which I referred above is the principle of molecular democracy:

"All enzymes are equal,
but some enzymes are more equal than others."


BOTTOM
27,000
28,000
41,000
NATIVE SUBUNIT
(42,000)
TOP
Time (min)
1
5
10
20
30
45
60

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